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CARL VOEGTLIN

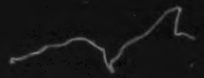
1879–1960

First Director of Cancer Research of the Public Health Service

and

First Chief of the National Cancer Institute

Bust by Margaret French Cresson
Stockbridge, Massachusetts,
October, 1940



DR. CARL VOEGTLIN died suddenly of a cerebral hemorrhage, on April 9, in Washington, D.C. With his death, an era in the National Cancer Institute ended.

Dr. Voegtlin was born in Zofingen, Switzerland. Soon thereafter his father, a doctor of medicine, moved the family to Basel, and it was here that Carl attended public school. In his autobiographical notes, he mentioned his happy memories of school vacations spent in the country, where he worked with farm boys and acquired a lifelong interest in nature. When he was nine years old, his mother died, and the next year his father, sister, and brother were killed in a railroad accident and he was taken into the family of an uncle.

At the age of 18 he entered the University of Basel, where he took courses in the preclinical sciences. At Basel he met the psychiatrist Carl Jung and other men who later became famous professionally. He left Basel to specialize in chemistry and physics at the University of Munich, where Bayer, Wieland, Willstäter, and Roentgen were working at the time. He then attended the University of Geneva and began his doctor's dissertation with Professor Graebe, who synthesized the first aniline dye—alizarine. His doctor's dissertation was completed under Professor Gatterman at the University of Freiburg, Germany, where he received his Ph.D. in 1904. Next he did organic chemical research in the laboratory of Professor William Perkin at Victoria University, Manchester, England.

Professor Graebe had advised him to go to the United States for a year. At the University of Wisconsin, offered an instructorship by Professor Daniels, head of the Department of Chemistry, Dr. Voegtlin taught qualitative and quantitative analysis. At the end of the year, when he was making plans for his return to Switzerland, Dr. L. F. Barker, head of the Department of Medicine at Johns Hopkins, asked him to start a biochemical research laboratory, the first in any Department of Medicine in the United States. At Johns Hopkins he was influenced by the example of Drs. Abel, Halsted, Howell, Kelly, Mall, and Welch. In 1908 he became an Associate of Dr. Abel in the Department of Pharmacology of Johns Hopkins, which he considered a turning point in his life. After a summer of research with Abderhalden in Berlin in the laboratory of Emil Fischer, he returned to Johns Hopkins and from 1908 to 1913 taught pharmacology there and continued in research.

In 1913 Dr. Voegtlin became Chief of the Division of Pharmacology at the U.S. Hygienic Laboratory, precursor of the National Institutes of Health. He was later commissioned as Pharmacologist Director with the rank of Medical Director in the U.S. Public Health Service. The National Cancer Institute Act went into effect on August 5, 1937, and on September 30, Carl Voegtlin was appointed Director of Cancer Research of the Public Health Service. In January, 1938, he was appointed first chief of the National Cancer Institute, and he held this position until his retirement in 1943. In October, 1943, he was appointed Consultant to the Medical Section of the Manhattan District. During the war and for several years thereafter he was Lecturer in Pharmacology at the University of Rochester, receiving an honorary Doctorate of Science for his contributions to the war and other research.

Dr. Voegtlin was a member of many scientific societies and served as Chairman or President of the American Society for Pharmacology and Experimental Therapeutics, the Federation of American Biological Societies, the Academy of Medicine of Washington, D.C., the American Association for Cancer Research, and the Section on Pharmacology and Therapeutics of the American Medical Association. He was a member of the Board of Directors of the American Society for the Control of Cancer. Other societies of which he was a member were the American Physiological Society, the American Society of Biological Chemists, the Harvey Society of New York, the Washington Academy of Science, the Society for Experimental Biology and Medicine,

the Clinicopathological Society of the District of Columbia, the American Association for the Advancement of Science, the Swiss Academy of the Medical Sciences, Phi Beta Kappa, and Sigma Xi. He acted as consultant and a member of such committees as the Board of Consultants of the Chemical Warfare Service, U.S. Army, the Committee on the Status of Intravenous Therapy of the American Medical Association, the first and second International Conferences on the Biological Standardization of Drugs, the Committee on Drug Addiction of the National Research Council, the Advisory Committee to the U.S. Secretary of Agriculture on the Toxic Action of Lead Arsenate Spray Residues on Apples and Vegetables, and Consultant of the medical section, Manhattan District, and Chief Toxocologist, University of Rochester Contract with the Manhattan District.

The titles of his publications are evidence of his wide knowledge and broad scientific interests. One of his early discoveries was the role of the parathyroid in the maintenance of blood calcium and its relation to muscular hyperexcitability in parathyroid tetany. During 1913–20, he contributed to knowledge of the relation of diet to pellagra, the toxicity of preservatives of antimeningitis serum, and the diagnosis and prevention of T.N.T. poisoning. When World War I cut off the sole source of supply of the arsphenamines from Germany, it was necessary to make these important drugs in this country, and Dr. Voegtlin was instrumental in working out toxicity tests for the regulations of the "Biologics Act" for the official control of the arsphenamines. This practical work led him to research on the fundamental aspects of the chemotherapeutic process or mode of action of the arsenicals. As a result he showed that Ehrlich's hypothetical "arsenic receptor" could be identified with the sulfhydryl group of certain cell constituents such as glutathione. The use of arsenic as a specific SH reagent led to the discovery of the so-called sulfhydryl enzymes in tissues.

From 1922 to 1940, Dr. Voegtlin's personal research work was largely confined to the biochemical aspects of cancer in which his expressed aim was to discover (1) any qualitative or quantitative differences in the chemical composition or behavior of tumors as compared with normal tissues, and (2) to add to our knowledge of the process of cell proliferation. These studies, in collaboration with many associates, included a comparison of the reducing power of normal and malignant tissue, the contents of glutathione and of ascorbic acid in tumor tissue, the effects of lysine, cystine, and methionine deficiencies upon the growth of tumors, the effect of oxygen tension and *p*H on the autolysis of normal and malignant tissue extracts and the apparent reversal of this process, and the conversion of normal cells into cancer cells *in vitro* by the action of methylcholanthrene.

As first Chief of the National Cancer Institute his whole energy was given to the direction of various activities of the Institute. These included planning the equipment of the building and the appointment of additional scientific and technical personnel. He was fortunate from the beginning in having a competent nucleus of personnel trained in cancer work, in the group in Washington and the group of Public Health scientists from Boston who joined the Staff. As director he gave maximum freedom to the scientific staff and encouraged cooperative research. To acquaint the staff with the many different aspects of the cancer problem, periodic meetings were held, frequently with outside lecturers. By 1940 the research output of the Institute had increased to such an extent that under provision of the National Cancer Institute Act he recommended and obtained annual appropriations for the publication of a bimonthly Journal of the National Cancer Institute. With the assistance of Miss Carolyn Whitlock, he edited the Journal until his retirement.

On his retirement from the Public Health Service in 1943, Dr. Voegtlin was still active as Consultant and Chief Toxicologist for the University of Rochester Project of the Manhattan District. Dr. Harold Hodges, in the introduction to *Pharmacology and Toxicology of Uranium Compounds*, 1949, wrote of Carl Voegtlin: "His guidance was obtained at every step. It is not too much to say that, without his mature knowledge of pharmacology and his experience as an administrator, the work reported in this volume would have gone haltingly indeed. His was a major con-

tribution." That particular study of the toxicology of uranium compounds represented the most comprehensive experimental investigation of an industrial poison ever carried out by any group of scientific workers in such a short time.

The varied activities and responsibilities of Carl Voegtlin as research worker and administrator did not limit his interests in many other aspects of life. He was an individual who quietly and wisely used every minute. He was a linguist and helped when translations were required. He was interested in art and music, interests that were fostered during his life in Munich. He was interested in governments, here and abroad.

In 1912 Dr. Voegtlin married Lillian Kreuter. His wife shared his interests in music, art, and the theater, and she was intensely interested in his scientific pursuits. Up to the week of his death, Mrs. Voegtlin was continuously the gracious hostess to small groups of friends and associates who spent many hours in exchanging points of view and listening to the thoughtful views of Dr. Voegtlin. These gatherings included many of the famous scientists of our time in addition to lesser known associates who thus were better able to become acquainted with the manysidedness of this remarkable man. He is survived by Mrs. Voegtlin and their son Hugh Stewart.

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On the Type of Hemoglobin in the Neoplastic Cell of Virus-Induced Fowl Erythroleukemia [1, 2]

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SUMMARY

The virus-induced fowl erythroleukemic cell may contain as much as one third of the hemoglobin content in the normal fowl erythrocyte (about 10 and 30 picograms of hemoglobin per cell, respectively). The hemoglobin prepared from erythroleukemic cells has been analyzed by paper- and starch-bloc electrophoresis and also with its alkali-denaturation kinetics. Erythrocyte hemoglobin preparations from normal birds and from the remaining normal erythrocytes in leukemic birds served as comparison material. The normal erythrocytes from both normal and leukemic birds showed two distinct hemoglobin components, I and II, of which component II accounts for 20 to 40 percent of the total. Hemoglobin prepared from erythroleukemic cells showed the same two types and in the same proportions as those of the normal erythrocyte hemoglobin. This indicated that the hemoglobin synthesis during the neoplastic cell development induced by the leukemia virus was under a similar genetic control as during the normal red-cell development.—J. Nat. Cancer Inst. 25:685–695, 1960.

MICROSPECTROPHOTOMETRY in the Soret region of single erythroleukemic cells revealed a specific absorption band at 415μ, which was interpreted to be due to hemoglobin-like compounds (*1*). These compounds are formed during neoplastic-cell development, which is induced by the leukemia virus in the bone-marrow erythroid stemcells of the host (*2, 3*). The following investigation describes some physicochemical properties of hemoglobin in the fully developed erythroleukemic cell and compares them with hemoglobin in normal fowls.

In the adult fowl, there are two distinct hemoglobin components: the main component (I) with a relatively low electrophoretic mobility at *p*H 8.8, and the second (II) with a much higher mobility (*4*). Component II accounts for about 20 percent (*5*). The difference in mobility between the two hemoglobin fractions can be explained by a rather large difference in the amounts of acid and basic amino acids (*5*).

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The fowl hemoglobin is thus relatively easy to identify with electrophoresis or other physicochemical means and provides a means of investigating whether or not the specific 415μ absorption and at the Soret region in the erythroleukemic cell is due to fowl hemoglobin I and II. The specific type of the erythroleukemic hemoglobin is interesting from another aspect. It is generally assumed that the formation of hemoglobin is genetically controlled (*6*). Cytogenetic changes in malignant tumor cells have been frequently described (*7*) and also the somatic mutation theory in virus-induced neoplasias has been discussed (*8*). The virus-induced erythroleukemic cell represents a neoplastic proliferating tumor cell, and the type of hemoglobin may indicate whether or not its development has been under abnormal genetic control.

MATERIALS AND METHODS

Six-month-old White Leghorn chickens of strain Edo were inoculated intravenously with high doses of erythroleukemic virus prepared according to earlier specifications (*9*). In the final stage of the disease (12 to 14 days after inoculation), and when the leukemic-cell counts in the peripheral blood showed sufficient high values (about 0.5 ml./mm.^{3}), heparinized blood was collected from the carotid arteries by decapitation. About 30 ml. of blood could be obtained from each animal.

For comparison, blood samples were obtained from healthy, 6-month-old chickens, as well as from normal embryos, at the 18th day of incubation. All the samples were subsequently treated by the following procedure for determination of hemoglobin.

The blood was spun in an MSE refrigerated centrifuge at 4° C., 500 $\times$ g for 20 minutes, which gave a distinct layering of the plasma, the leukemic cells, and the erythrocytes. The layers were separated with capillary pipettes. Only the central parts of the cellular layers were collected.

The purity of the fractions was checked by smears of small aliquots that were stained by the May-Grünwald-Giemsa procedure (fig. 1). Total and differential cell counts were made, and the hemoglobin concentrations of the respective lysates were determined by spectrophotometry. From these values, the average hemoglobin content per cell of the different fractions was calculated.

The erythrocyte and leukemic-cell fractions were washed 3 times with physiological saline, lysed with 10 volumes of distilled water, and then subsequently frozen at $-20°$ C. Optically clear hemoglobin solutions were obtained by centrifuging the thawed lysates at 10,000 $\times$ g for 20 minutes. The cyanide compound (HbCN) was made with potassium-cyanide and -ferricyanide, according to reference (*10*). The various solutions from erythrocytes and leukemic cells were diluted with distilled water, to contain about 5 gm. HbCN per 100 ml. (text-fig. 1).

The following methods were used to characterize the hemoglobin obtained from the erythrocytes and the leukemic cells:

1. *Electrophoresis on starch* at high voltage was made according to the

method described by Wieland and Pfleiderer (*11*, *12*), with an apparatus manufactured by Hormuth and Vetter Ltd., Heidelberg. Amylum solubile (Merck), 120 gm., was saturated with a 0.06 N Veronal-buffer solution, *p*H 8.6, to form a thin bloc of 14 × 30 cm.² area.

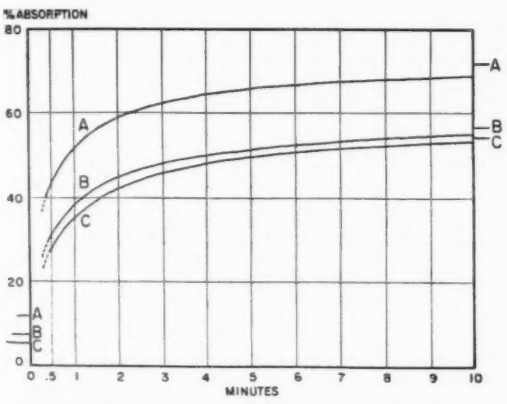
A few tenths of a milliliter of the HbCN solution (cyanide compound) was put on the starch bloc as a "starting line" and the electrophoresis was made at 1750 v., 50 ma., for 2 to 4 hours. The cyanide derivative showed good stability and prevented the formation of methemoglobin, which has somewhat different electrophoretic properties and could otherwise lead to artifacts and misinterpretation (*13*).

2. Separation was also made with the *paper electrophoresis* apparatus manufactured by LKB, Stockholm. The buffer was a 0.05 M Veronal-acetate solution, *p*H 8.6. For 12 to 15 hours, 250 v., 10 ma. was applied.

3. *Reaction kinetics of the denaturation* of the HbCN by sodium hydroxide were made, in principle as described earlier (*10*, *14*), but with some technical modifications.

The reaction cyanide-hemoglobin→ alkaline hematin was brought about by the addition of 4.5 ml. of 0.1 N NaOH to 0.5 ml. of the HbCN solution in a 1 cm. cuvette. The reaction kinetics were continuously recorded on a constant-speed chart as the increase of absorption at the wavelength of 640μ.

The recording spectrophotometer was in principle similar to the description in reference (*1*) but especially adapted for work in the "macro"- cale. The stability for a period of 15 minutes was comparable to an absorbency $\left(\log \frac{I_0}{I_1}\right) < 0.001$. Thus the reaction kinetics of the denaturation of hemoglobin could be recorded with high accuracy.



TEXT-FIGURE 1.—Example of spectrophotometer records of changes in light absorption at 640 mμ during the alkali denaturation of hemoglobin from (A) erythrocytes from normal bird, (B) erythroleukemic cells, and (C) erythrocytes from embryos. The absorption values at start of the reaction indicated *extreme left*, the values at end of the reaction (15 minutes) *right*—experiment 10.

The percentage of the undenatured hemoglobin at each point of time during the reaction could be calculated from:

$$Hb_t = \frac{E_t - E\infty}{E_t - E_o} \text{ const.}$$

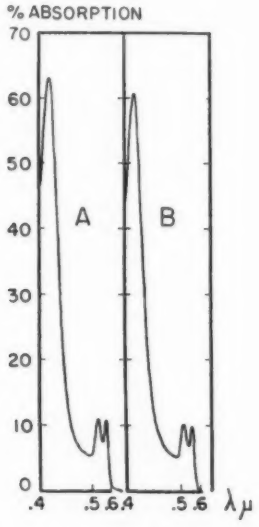
where E_o = the absorbancy at the start of the reaction
$E\infty$ = the absorbancy at the end of the reaction
and E_t = the absorbancy at time t during the reaction.

The logarithm of Hb_t is a function approximately linear with time. The proportions between the differently reacting types of hemoglobin can be obtained by extrapolating this function to zero time.

As a complement to the optical procedure, the precipitation method of Singer *et al.* (*15*) was used. Determinations of the soluble HbCN remaining at different time intervals after the alkali treatment were made spectrophotometrically at the wavelength of 574 mμ.

RESULTS AND DISCUSSION

Table 1 shows quantitative analyses of hemoglobin prepared from erythroleukemic cells and normal erythrocytes, respectively. The mean leukemic-cell hemoglobin can be as high as one third the normal erythrocyte hemoglobin value. Spectral absorption curves of hemoglobin from the two different sources are practically identical, as demonstrated by text-figure 2. This shows that the absorption band at 415 mμ, which can



TEXT-FIGURE 2.—Spectrophotometer records of hemoglobin prepared from (A) normal erythrocytes and (B) erythroleukemic cells. Wavelength range 400 to 600 mμ—experiment 5.

TABLE 1.—Estimation of hemoglobin content of erythroleukemic cells as percentage of normal-fowl erythrocyte hemoglobin (expts. 5 and 11)

	Erythrocyte suspension	Leukemic-cell suspension
Total cell number		
Expt. 5	18.0×10^9	7.1×10^9
Expt. 11	27.2×10^9	5.25×10^9
Erythrocyte percentage		
Expt. 5	100	6
Expt. 11	100	20
Light absorption $\left(\log\frac{I_0}{I_1}\right)$ ratios at 546 mμ of extracted hemoglobin (same total volumes)		
Expt. 5	6.5	1
Expt. 11	10.0	1
Mean leukemic-cell hemoglobin in percentage of erythrocyte hemoglobin		
Expt. 5	35	
Expt. 11	39	

be detected by microspectrophotometry in the single erythroleukemic cell (*1*), is indeed due to hemoglobin.

During the preparation of the different hemoglobin samples from leukemic cells, great care was taken to minimize contamination with the normal erythrocyte hemoglobin. In calculating from the differential counts in table 2, this contamination in most cases does not exceed 20 percent. The analyses with electrophoresis and alkali-denaturation kinetics can therefore be regarded as representative of the hemoglobin derived either from erythroleukemic cells or from normal erythrocytes.

The electrophoretic analyses exemplified by figure 2 confirm the earlier findings (*4*, *5*) that the normal fowl hemoglobin has two distinguishable components. A third component, which Rodnan and Ebaugh reported as occurring in a few "mongrel Leghorns" (*16*), could not be seen. The same electrophoretically defined components with equal mobilities

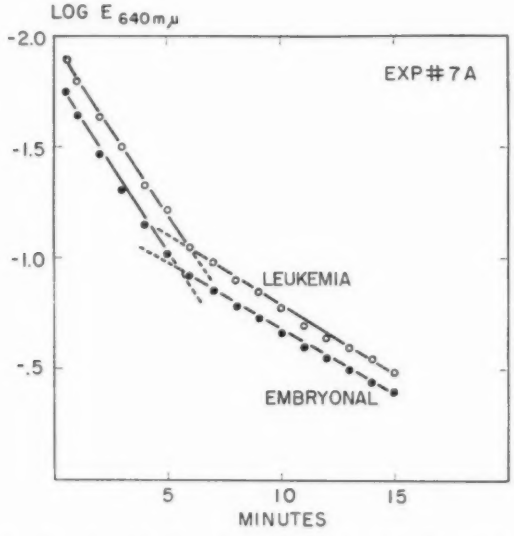
TABLE 2.—Differential counts of leukemic cells in peripheral blood of chickens inoculated with erythroleukemia virus*

Expt. animal No.	Leukemic-cell counts (*mill./mm.*^{3}) in the peripheral blood	Erythrocyte percentage in the purified leukemic-cell preparation
1	0.60	14
2	0.50	8
4	0.45	6
5	0.20	6
7	0.55	5
10	0.45	15
11	0.35	20

*Twenty chickens were inoculated with erythroleukemia virus. The blood of 15 chickens at the final stage of disease (10–15 days after inoculation) showed well-developed leukemic cells according to (*8*). Only those samples showing a relatively pure leukemic-cell fraction, as exemplified by figure 1, were used for the subsequent hemoglobin analyses.

found in the hemoglobin samples prepared from normal erythrocytes were found in hemoglobin from erythroleukemic cells. No abnormal hemoglobin component could be detected.

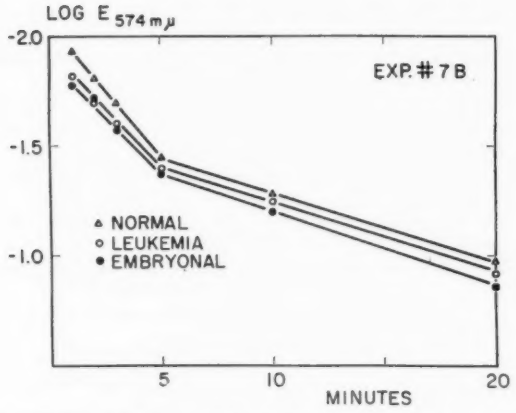
Text-figures 3 and 4 show the results of typical alkali-denaturation experiments. All analyses reveal two distinguishable components, which are the same for hemoglobin prepared from erythroleukemic cells as for normal erythrocyte hemoglobin. Within the error of the method, component II accounts for about 20 to 40 percent, which does not vary significantly between samples from the different sources.



TEXT-FIGURE 3.—Reaction kinetics of the alkali denaturation of hemoglobin from erythroleukemic cells and embryonal erythrocytes measured with the direct spectrophotometric method. Example of a typical experiment (7A)—*see* text.

The results confirm and extend earlier findings (*1*) that hemoglobin is formed by the erythroleukemic cell. As hemoglobin synthesis is the characteristic process during the normal differentiation of the erythroid bone marrow cell, this gives the neoplastic erythroleukemic cell certain properties of differentiation. The pathological development, which is induced by the virus and which leads to the typical leukemic cell, has been defined by cytological methods (*2*). The present experiments analyze the physicochemical specificity of the hemoglobin synthetized during this development.

A number of investigations have established the important contribution of genetic factors in giving rise to physically different hemoglobin molecules in man. The differences are ascribable to inherited alterations in the globin portion. Although the genetic situation regarding the fowl hemoglobin I and II is not as well known as that of the different human



TEXT-FIGURE 4.—Reaction kinetics of the alkali denaturation of hemoglobin from the three sources indicated, measured with the precipitation method. Example of a typical experiment (7B). Log E is calculated for the denaturated hemoglobin to conform with text-figure 3.

types of hemoglobin, the two hemoglobin components must be presumed to represent a species-specific, genetically controlled heterogeneity of the hemoglobin molecules.

A cytogenetic alteration during the virus-induced leukemic-cell development might give rise to an abnormal type of hemoglobin. The existence of an additional hemoglobin component does not necessarily indicate that a mutant gene is responsible, however. Modifications due to a changed physiological condition of the cell during hemoglobin formation can give secondary changes after the initial protein synthesis.

The present investigation showed no significant differences between the hemoglobin from normal red cells and from erythroleukemic cells. This is somewhat surprising in view of the two different types of cells from which the hemoglobin samples originated. However, a supposedly normal cytogenetic situation, as reflected by the synthesis of the normal fowl hemoglobin I and II, during the erythroleukemic-cell development, agrees in principle with the findings that these leukemic cells exhibit the normal, diploid macrochromosome constitution of the chicken (*17*). It might even be that any change in the genotype of the infected cell will upset the established cell-virus relationship and lead to a selective disadvantage and elimination.

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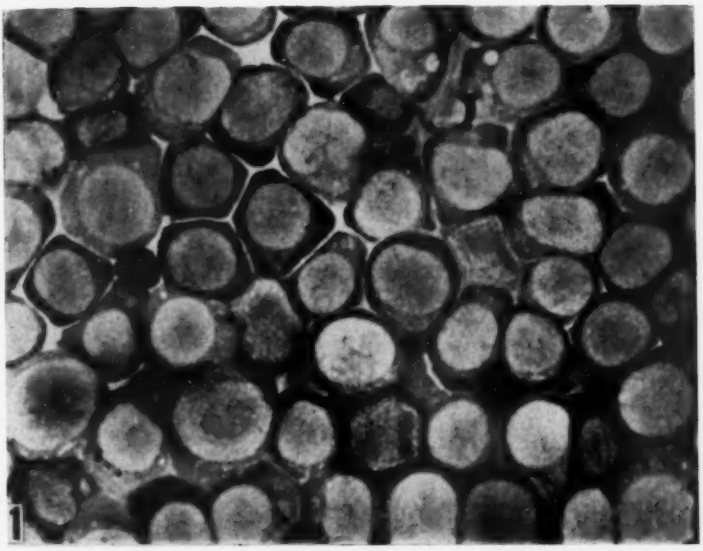
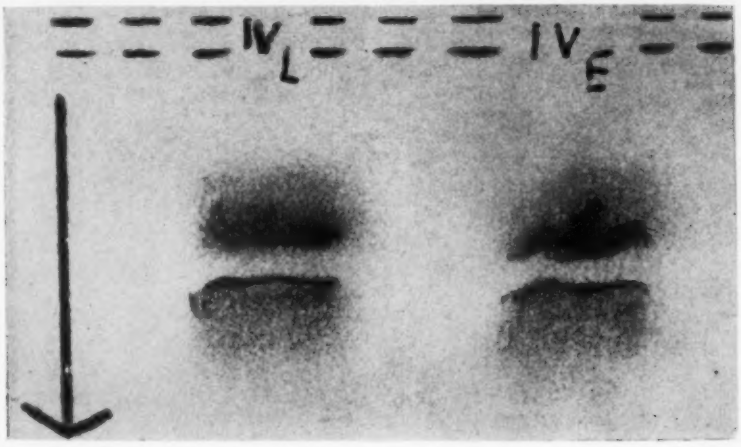
PLATE

PLATE 103

FIGURE 1.—Smear of a leukemic-cell fraction from which hemoglobin was prepared (expt. 2). Giemsa stain. × 1,000

FIGURE 2.—Electrophoretic separation on starch, of the two fowl hemoglobin components: Leukemic-cell hemoglobin, *left*, normal erythrocyte hemoglobin, *right*. Starting zone between the dotted lines (expt. 4), *see* text.





560070—60——3

Distribution of Radioactivity After Intravenous Administration of 7,12-Dimethylbenz[*a*]anthracene-7,12-C^{14} Into the Mouse [1, 2]

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SUMMARY

Within 30 seconds after the intravenous injection of C^{14}-labeled 7,12-dimethylbenz[*a*]anthracene, radioactivity was found in mouse skin, blood, liver, kidney, and spleen. The proportion of radioactivity carried by precipitated protein changed with time. These findings did not rule out a direct carcinogenic effect on mouse skin by 7,12-dimethylbenz[*a*]anthracene or its metabolites after the intravenous administration of the polynuclear hydrocarbon.—J. Nat. Cancer Inst. 25: 697–701, 1960.

THE BIOLOGICAL activity of the potent contact carcinogen 7,12-dimethylbenz[*a*]anthracene after direct application to mouse skin in large (*1*) and small amounts (*2–4*) has been extensively investigated. The intravenous injection of the polynuclear hydrocarbon has also been reported to initiate carcinogensis in mouse skin (*5*). This observation by Graffi, Scharsäch, and Heyer pointed to the possibility that 7,12-dimethylbenz[*a*]anthracene was carcinogenic because of some indirect mechanism. An indirect mechanism for the carcinogenic action of 7,12-dimethylbenz[*a*]anthracene also appeared possible in view of older data obtained with C^{14}-labeled dibenz[*a,h*]anthracene of low specific activity (*6*). Heidelberger and Jones (*6*) found that no radioactivity was detectable in mouse skin 24 and 48 hours after the intravenous injection of labeled dibenz[*a,h*]anthracene. The localization of radioactivity in mouse skin and several other tissues, after the intravenous injection of 7,12-dimethylbenz[*a*]anthracene-7,12-C^{14} of high specific activity, has now been investigated.

The radioactivity carried by precipitated protein derived from the various tissues was also measured. Although it was not known to what extent this was an *in vivo* process or one induced during the disruption (*7*) of the tissue, this empirical measurement served to detect differences in the radioactive content of the tissues investigated.

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[2] This work was supported by grant C-2399 and cancer control grant CS-9212, from the National Cancer Institute, National Institutes of Health, Public Health Service, and grant L-4B from the American Cancer Society, Inc.

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560070—60——4

EXPERIMENTS

Administration of the Carcinogen

Virgin, female Swiss mice in the second resting phase of the hair cycle were similar to those used previously (*3*). The 7,12-dimethylbenz[*a*]-anthracene-7,12-C^{14} (*8*) was injected intravenously into the tail vein of the mouse as an aqueous colloidal solution, prepared at 65° C., and stabilized by 1 percent gelatin (*6*, *9*). The preparation of the colloid has been recently described in detail (*10*). After the injection was completed, the tail was wiped with cellulose tissue, previously moistened with acetone to remove any exposed hydrocarbon. The backs of the mice were shown to be fluorescence-free.

At the appropriate time, the mice were decapitated, and the blood was collected and stored in a centrifuge tube at dry-ice temperature. The various excised tissues, except skin, were wrapped in aluminum foil and stored in a thermos flask filled with dry ice until they were processed. The skin was processed immediately (*3*). The centrifugal separation of the whole homogenate into supernatant and particulate fractions was eliminated in tissues other than skin, and a single fraction of protein was precipitated and purified, as described previously for the two skin fractions (*3*).

Radioactive Assay

Samples for radioactive assay were counted in an internal flow counter, were corrected for self absorption, and were statistically significant to at least 10 percent. The samples were prepared as described previously (*3*) except that the preparation of the protein plate was improved. The air in the vicinity of the plate was ionized by a static eliminator (source of α-rays), and thus dissipated the electrostatic charges on the protein particles. This greatly facilitated the handling of the dry protein, the coalescence of the protein particles in the aqueous alcohol suspension, and the adherence of the protein particles to the plate.

RESULTS AND DISCUSSION

Within 30 seconds after the intravenous injection of the colloid, there was radioactivity in mouse skin (table 1). At this early time only the precipitated supernatant protein fraction carried radioactivity. During the remainder of the 1st day, both the particulate and supernatant protein fractions carried radioactivity. At the 7th day radioactivity could be detected only in the particulate fraction of the skin. These observations were of the same general pattern as those noted when 7,12-dimethylbenz[*a*]anthracene was applied directly to the skin (*3*).

A dose of 1 μg. of 7,12-dimethylbenz[*a*]anthracene per 3.5 cm.^{2} of mouse skin was sufficient to act as a potent initiator of carcinogenesis (*3*, *4*). This dose corresponded to 36,400 counts per minute (cpm) for 4 standard circles of excised skin (*3*). At all times, after the intravenous

injection of 350 μg. of labeled hydrocarbon per mouse, a lesser amount of radioactivity was found in the whole homogenate of the skin. The relative localization of radioactivity in the mouse skin became reversed by the time 1 day had elapsed. The whole homogenate, of 4 excised circles of skin, now contained 440 and 1010 cpm, respectively, after the direct application of 1 μg. of labeled carcinogen per 3.5 cm.^{2} of mouse skin or the injection of 350 μg. of labeled carcinogen per mouse (table 1). Thus the direct carcinogenic action of 7,12-dimethylbenz[*a*]anthracene or its metabolites in the experiments of Graffi, Scharsäch, and Heyer (*5*) was not ruled out by these data.

When these results are compared with those obtained with dibenz[*a*,*h*]-anthracene (*6*), it should be noted that the specific activity of the labeled 7,12-dimethylbenz[*a*]anthracene was about 20 times that of the labeled dibenz[*a*,*h*]anthracene. There was also the possibility that radioactivity might have been detected in the skin, before the elapse of the 1st day, after the injection of labeled dibenz[*a*,*h*]anthracene.

Radioactivity was found in the blood, liver, kidney, and spleen as well as skin after the intravenous injection of 7,12-dimethylbenz[*a*]anthracene-7,12-C^{14} (table 2). Although there was considerable radioactivity in the spleen and kidney, 30 seconds after the injection, the precipitated protein derived from these organs did not carry detectable amounts of radioactivity at this time. During the 1st day, the radioactivity carried by the precipitated protein was the most persistent radioactivity in the tissues examined (table 3). At the end of 1 week, this persistence had: (1) become more prominent with the particulate fraction of the skin, blood, and kidney; (2) become less prominent with the supernatant fraction of the skin and liver; and (3) remained unchanged with the spleen (table 3).

TABLE 1.—Radioactivity in mouse skin after intravenous injection of 7,12-dimethylbenz[*a*]anthracene-7,12-C^{14}*

	Time after injection:				
	30 seconds	30 minutes	17 hours	1 day	1 week
Whole homogenate					
Wet weight (mg.)	196	178	199	204	214
Dry weight (mg.)	57	63	84	67	58
Total activity (cpm)	4550	5490	2200	1010	TLM†
Particulate protein					
Dry weight (mg.)	27	20	22	23	26
Total activity (cpm)	TLM	124	98	92	121
Specific activity (cpm/mg.)	TLM	6	4	4	5
Supernatant protein					
Dry weight (mg.)	4.4	4.1	3.2	4	3.8
Total activity (cpm)	27	95	53	33	TLM
Specific activity (cpm/mg.)	6	23	17	8	TLM

*Each determination was made on the pooled tissue of 4 animals. Two circles of skin, 2.5 cm.^{2} in area, were excised from each animal. The results were tabulated on a basis of 4 circles of skin and are directly comparable to the tables in (*5*). The injected dose was 350 μg. of hydrocarbon (4,340,000 cpm) in 0.5 ml. of colloid per mouse.

†TLM = too low to measure.

TABLE 2.—Localization of radioactivity in mouse tissue after intravenous injection of 7,12-dimethylbenz[*a*]anthracene-7,12-C^{14}*

	Time	Tissue			
		Blood	Liver	Kidney	Spleen
Whole homogenate, wet weight (mg.)	30 sec.	†	5, 580	904	1, 250
	30 min.	1, 670	4, 140	860	501
	17 hrs.	1, 520	4, 570	920	420
	1 da.	1, 660	4, 100	965	365
	1 wk.	1, 670	5, 080	1, 130	537
Whole homogenate, dry weight (mg.)	30 sec.	†	1, 070	238	257
	30 min.	305	1, 330	234	122
	17 hr.	302	1, 510	276	104
	1 da.	291	1, 310	267	101
	1 wk.	354	1, 550	307	125
Whole homogenate, total activity (cpm)	30 sec.	†	2, 420, 000	293, 000	209, 000
	30 min.	68, 900	1, 280, 000	186, 000	59, 200
	17 hr.	8, 040	287, 000	19, 000	5, 560
	1 da.	4, 720	232, 000	11, 000	3, 050
	1 wk.	1, 530	68, 800	3, 140	2, 950
Precipitated protein, dry weight (mg.)	30 sec.	†	1, 040	141	168
	30 min.	140	841	138	65
	17 hr.	133	873	160	56
	1 da.	180	797	158	51
	1 wk.	188	880	187	71
Precipitated protein, total activity (cpm)	30 sec.	†	7, 240	TLM ‡	TLM
	30 min.	1, 170	91, 300	2, 500	441
	17 hr.	1, 380	79, 100	2, 480	445
	1 da.	1, 440	71, 700	1, 760	300
	1 wk.	890	18, 100	649	121
Precipitated protein, specific activity (cpm/mg.)	30 sec.	35	7	TLM	TLM
	30 min.	8	110	18	7
	17 hr.	10	91	17	8
	1 da.	8	90	11	6
	1 wk.	5	21	4	5

*The data in tables 1 and 2 were obtained from the same animals. The results were from the pooled tissue of 4 mice.

† This value was not determined because of the short experimental time. Sufficient blood was collected to calculate the data in table 3. In the blood collected there were 940,000 cpm and the protein carried 6,600 cpm.

‡ TLM = too low to measure.

TABLE 3.—Percentage of radioactivity in tissue carried by precipitated protein*

	Time after injection:				
	30 seconds	30 minutes	17 hours	1 day	1 week
Skin (particulate)	0	2. 2	3. 6	9. 1	100
Skin (supernatant)	0. 6	1. 7	2. 4	3. 2	0
Skin (whole)	0. 6	3. 9	6. 9	12. 4	100
Blood	0. 7	1. 7	17	30	58
Kidney	0	1. 3	13	16	21
Spleen	0	1. 5	8	9. 8	9. 8
Liver	0. 3	7. 1	27	31	26

*These data were calculated from tables 1 and 2.

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Modified Drop Technique: Preparation of Smears for Automatic, Electronic Scanning [1, 2]

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SUMMARY

A modified application of the "drop technique" for cytology and its desirability for automatic, electronic scanning are discussed. Vaginal pool aspirates like all cytological specimens frequently have sparsely populated cellular material, and when the Cytoanalyzer is used, an optimum sampling of well-prepared specimens is desirable. Specimens are obtained by aspiration from the posterior vaginal fornix and suspended in 75 percent ethanol for fixation. This suspension is poured through a fine sieve and centrifuged; the supernatant is removed, the cellular material is diluted to a known density, and a known quantity of the specimen is dropped on a slide and spread over a previously defined area with the tip of the pipette. Feulgen staining is used to obtain a nuclear stain of medium intensity with minimal staining of the cytoplasm. A coverslip is mounted with Permount and the slide is ready for automatic scanning. Processing by this method provides an adequate control of the number, spacing, and distribution of cells in the smear without distorting their morphological characteristics.—J. Nat. Cancer Inst. 25: 703–711, 1960.

THE CYTOANALYZER, an experimental computing instrument, is designed to separate uterovaginal smears into two groups, negative and questionable (*1*), and by measuring the physical parameters of nuclear size and density rejects or records cells in different zones on the basis of mathematical relationships to model nuclear parameters applied by the operator. Certain categories of cells are designated as normal and others as abnormal. From the number of entries in each zone, a ratio of abnormal cells for each smear is established and the smear is classified as negative or questionable. All questionable specimens must be screened

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[4] The authors wish to thank Miss Sarah Jane Smith and Mr. Paul M. Montague, senior technicians, for their help, and Mr. Andrew K. Coffman and Dr. W. Ross Cameron for their cooperation in providing space for this research. The laboratory staff of the Columbus, Ohio, Cancer Survey Project provided positive and negative specimens and follow-up studies. The Departments of Gynecology at Johns Hopkins Hospital and Walter Reed Army Hospital supplied positive specimens.

[5] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

by cytotechnicians and/or cytologists. Personnel experienced in screening Feulgen-stained smears can screen the same smears scanned by the Cytoanalyzer. A smear made by the standard technique (*2*) can be prepared at the same time the specimen for the Cytoanalyzer is collected and the standard smear screened when indicated.

For satisfactory results from the Cytoanalyzer, a smear must (*a*) contain a portion of the specimen generous enough to provide an adequate sampling of all the cell types present; (*b*) have a concentrated distribution of the specimen along the longitudinal axis of the slide to enable the automatic focus to function properly; (*c*) have a monolayer of the cells on the slide; (*d*) show individual epithelial and leukocyte nuclei separated by a distance of more than 2μ; (*e*) be stained by a process that will not stain erythrocytes but will provide minimal staining of the cytoplasm and moderate staining of the nucleus; and (*f*) be processed by a technique causing minimal cellular distortion.

A semiautomatic mechanism has recently been installed on the Cytoanalyzer so that the optical system will remain in optimum focus. Focusing is accomplished by use of a spring-loaded stage which presses the slide firmly against two feet secured to opposite sides of the microscope objective and adjusted to a distance that will maintain the top surface of the microscope slide in the focal plane. Thus, while the slide moves back and forth beneath the objective, the cells will stay in focus. With this setup more than 95 percent of the cells remain within $\pm 3\mu$ of the focal plane (*3*). Focusing errors of greater than 3μ may result in significant errors in cell measurements.

For proper functioning of the automatic focusing device the feet must be able to glide freely on the top surface of carefully cleaned slides and the coverslip should not interfere with their movement. Since the slide moves back and forth longitudinally beneath the feet, the specimen must be spread and mounted over a defined area along the longitudinal axis of the slide. Specially sized coverslips (6 $\times$ 65 mm.)[6] are mounted over the smear which has been placed on a 25 $\times$ 75 mm. slide (text-fig. 1). This method allows the smear to cover approximately 4 cm.^{2} on each slide and provides an ample area for an adequate sampling of the specimen.

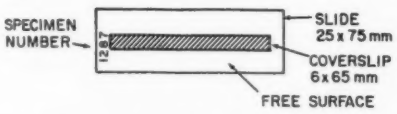
METHODS

Collection.—The uterovaginal specimens are obtained by blind aspiration of secretions in the posterior vaginal fornix. Standard vaginal pipettes are used, with a 2 ounce rubber bulb as the aspirating force. The aspirate is evacuated into 40 cc. of 75 percent ethanol and immediately shaken to disperse the cells—75 percent ethanol affords a relatively slow fixation allowing the cells to be dispersed and suspended in the solution before fixation, which prevents noticeable cellular degeneration even though left at room temperature for as long as 3 weeks. Other fixatives

[6] Clay Adams #1 Gold Seal A 1420, Beth-Mont Surgical Supply Co., Bethesda, Md.

were unsatisfactory for our purposes for several reasons: (1) too rapid fixation of the secretions entrapping the cells within the mucus, (2) distortion of cells, and/or (3) inadequate preservation of the specimen.

Processing.—The ethanol suspension is poured through a 53 μ pore-size sieve to remove the larger artifacts, strands of mucus, and clumps of cells so that there will be minimal variation in size of the particles, and then transferred to centrifuge tubes [7] and centrifuged at 140 $\times$ g (750 rpm) for 5 minutes. Experiments show that this centrifugal force is almost the minimum time required to recover most of the cells (text-fig. 2).



TEXT-FIGURE 1.—Diagram of a slide prepared for the Cytoanalyzer.

R.C.F./225 X G			140 X G			100 X G			70 X G		
Spec.	Avg. cells/ 10 h.p.f.	Overall avg.	Spec.	Avg. cells/ 10 h.p.f.	Overall avg.	Spec.	Avg. cells/ 10 h.p.f.	Overall avg.	Spec.	Avg. cells/ 10 h.p.f.	Overall avg.
1.	27.1	16.2	5.	22.1	9.1	9.	36.1	22.3	13.	Too	
2.	12.3		6.	9.1		10.	9.5		14.	numerous	
3.	19.4		7.	1.3		11.	24.1		15.	to	
4.	5.9		8.	3.9		12.	19.5		16.	count	

TEXT-FIGURE 2.—Sixteen generous vaginal aspirates suspended in 75 percent ethanol were centrifuged at the designated relative centrifugal force. After centrifugation the supernatants were poured through separate # SM Millipore filters, the cells in the filters were stained by standard techniques, and those in 10 high-power fields were counted.

Titration and smearing.—After centrifugation the supernatant is poured off and the cellular material is transferred with a pipette to a 10 $\times$ 75 mm. cuvette. The cuvette is inserted into a densitometer [8] with the potentiometer maintained at a setting of 50 percent *T*. The suspension is diluted with 75 percent ethanol until a light transmission of 40 $\pm$ 2 percent is registered on the direct-reading galvanometer; then 0.1 ml. is removed, dropped on the center of the slide, and spread uniformly with the tip of the pipette [9] over a previously defined area. The smears are air-dried before immersion in an acetic-formalin solution to await staining.

Slides.—Slides 25 $\times$ 75 mm. with plain unfrosted ends are used for the smears. Most slides have surface waves and variations in thickness which mildly affect the operation of the automatic focus. To minimize these variations, specially selected slides [10] of 3 to 6 μ are used.

[7] Model BE Centrifuge, Standard 16-place Head with 50 ml. shields. International Equipment Company, Boston, Mass.

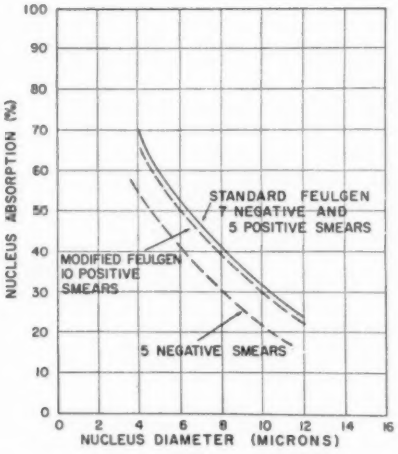
[8] Coleman Model 9 Nepho-colorimeter.

[9] Disposable pipettes for this purpose can be obtained from the Fischer and Porter Company, Towson, Md.

[10] Capital Surgical Supply, Harrisburg, Penna.

As previously mentioned, the objective feet must move freely on the clean lateral surfaces of the slide. Scotch tape is used to confine the smear to the 6 mm. center strip running the length of the slide. The slides are labeled at one end with a diamondpoint pencil, cleaned, and taped, before a specimen is dropped on them. After the smears are dry, the Scotch tape is removed, and they are placed in the acetic-formalin solution. Other methods of confining smears are being investigated, but none have proved as satisfactory as the method described.

Staining.—Since the Cytoanalyzer sorts cells on the basis of nuclear area and optical density, a staining reaction is desirable which supplies moderate nuclear and minimal cytoplasmic staining. Feulgen staining technique most nearly fulfills these requirements—it is possible



TEXT-FIGURE 3.—Differential staining of smears—median epithelial-cell nucleus absorption versus diameter of 200 consecutive cells from premenopausal smears stained by standard and modified Feulgen reaction. Positive and negative smear characteristics for the standard stain fall on the same line, while for the modified Feulgen there is a distinct separation.

to vary the staining by modifying the strength of the reagents and the application time. The traditional method of Feulgen staining (*4*) has two properties which produce undesirable results in the smear: (*a*) sporadic "precipitation" of dye in the cytoplasm, producing a granular appearance, and (*b*) a variable pink stain to the cytoplasm which varies with individual specimens. Accordingly a shape and density may develop which are measured as an abnormal nucleus, and this may cause an entry into a zone theoretically reserved for abnormal epithelial-cell nuclei. The screening ability of the machine may thus be less effective. By varying the staining time and the concentration of the dye, undesirable effects are eliminated, and the degree of nuclear staining is reduced.

Further reduction of staining time and/or dye concentration significantly reduces the nuclear staining reaction.

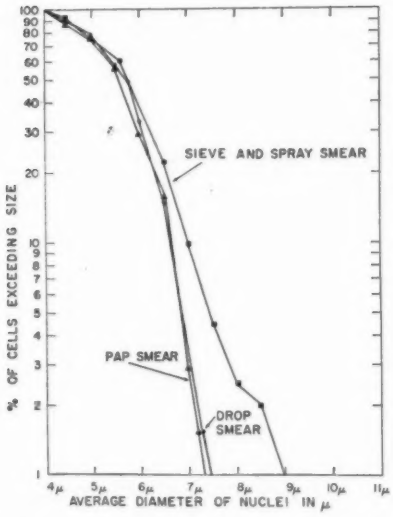
With a modified Feulgen reaction there seems to be a differential staining index for positive and negative premenopausal specimens. Ten consecutive positive smears, from untreated patients, stained more deeply than five consecutive negative smears (text-fig. 3). This phenomenon appeared in premenopausal specimens but less often, or not at all, in a comparable number of postmenopausal specimens. A possible explanation is the presence of relatively enlarged nuclei which are characteristic of malignant cells and also present to a lesser degree among nonmalignant cells in postmenopausal smears.

The stain most suitable for our purposes is the Feulgen reaction. Schiff's reagent is prepared by the traditional method (*5*) except that only 0.5 gm. of basic fuchsin per 100 cc. of distilled water is used instead of the usual 1.0 gm. per 100 cc. The staining procedure is: (*a*) Place slides in preheated distilled water at 60° C. for 1 minute. (*b*) Transfer slides to 1 N HCl for 10 minutes at exactly 60° C. (*c*) Transfer slides to Schiff's reagent for 10 minutes at room temperature. (*d*) Transfer slides to distilled water for 1 minute. (*e*) Transfer slides to acid-alcohol for 10 minutes. (*f*) Transfer slides to absolute alcohol for 1 minute. (*g*) Clear slides in xylene for 5 minutes. The smears are then mounted with Permount and set aside to dry before being prescreened. The cell nuclei appear reddish purple and the cytoplasm is usually not seen because it has absorbed practically no stain and its index of refraction closely approximates that of Permount; refractive index of cytoplasm is 1.54 and that of Permount 1.517 (*6*).

RESULTS

Previous smears for the Cytoanalyzer were prepared by the sieve-and-spray technique (*7*) which provided good spacing between cells. However many of the cells in the specimen were lost in the process of spraying and an undue number of specimens had to be declared unsatisfactory because of the paucity of material in the smear. This was not entirely due to the spray technique but was frequently caused by the small amount of material in the specimen sent to the laboratory.

Microphotometer analysis of the smears revealed considerable distortion of the nuclei. The major structural components of the nucleus remained intact and macroscopically there appeared to be no distortion (figs. 1, 2, 3, and 4). Actual measurements revealed distortion with cross-section enlargement of 15 to 20 percent (text-fig. 4) in nuclei that should normally measure only 7 to 8 μ in average diameter. This flattening of the nucleus also causes decreased optical density of the affected nuclei. The severity of this effect is not the same for all specimens measured but many smears have shown distortion similar to the smear presented in text-figure 4.



TEXT-FIGURE 4.—Comparison of nuclei prepared by different methods: The standard Papanicolaou method, the drop technique, and the sieve-and-spray technique. Three specimens were taken from each patient. From each smear, 200 consecutive epithelial-cell nuclei were measured and plotted on the graph.

DISCUSSION

Preparation of smears by the modified drop technique provides good control of the number, spacing, and distribution of cells without distorting the cell nuclei. However, even with this technique, a number of specimens must be called unsatisfactory because of the paucity of cellular material. After these particular specimens are sieved and centrifuged, it is apparent that almost all the material will be necessary to make an adequate smear. In such cases the suspension is diluted with a minimal amount of 75 percent ethanol until the visual density approximates a known standard. Then 0.1 ml. of the suspension is spread on the slide by the previously described method. A clinical trial of 10,000 specimens processed by the technique described is now in progress to test the potentialities of the Cytoanalyzer in automatically prescreening smears.

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PLATE

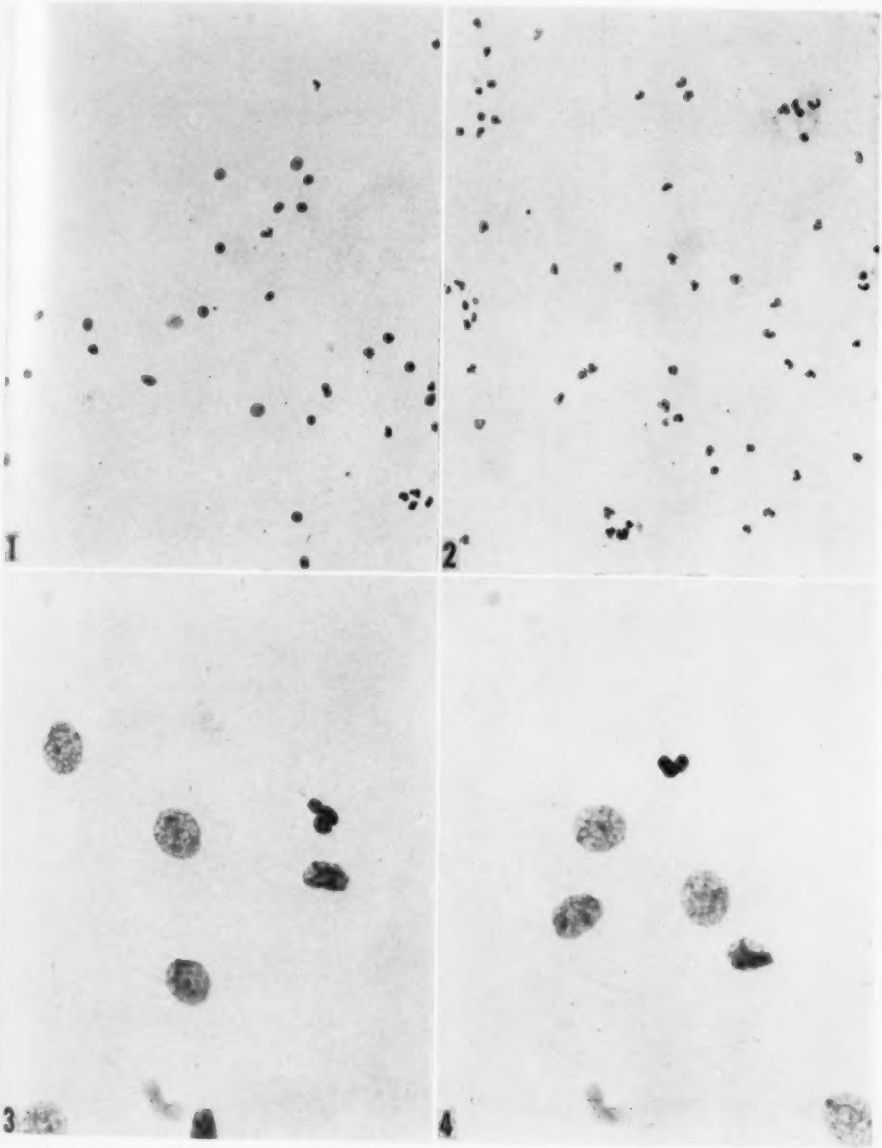
PLATE 104

FIGURE 1.—Distribution of cells—modified drop technique. Feulgen. × 310

FIGURE 2.—Distribution of cells—spray technique. Feulgen. × 310

FIGURE 3.—Morphology of cells—modified drop technique. Feulgen. × 1,000

FIGURE 4.—Morphology of cells from same patient as in figure 3; spray technique. Feulgen. × 1,000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Indications of Involvement of the Reticuloendothelial System in Alterations of Iron Metabolism Produced by Tumor Extracts [1]

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SUMMARY

Small, repeated, subcutaneous injections of an extract of the Walker carcinosarcoma 256 into normal rats maintained liver and kidney catalase activity and plasma-iron concentration at the low level usually found in tumor-bearing animals. These extracts also stimulated the phagocytic activity of the reticuloendothelial system (RES). Zymosan, a known stimulator of the RES, produced many of the same changes as those found when tumor extracts were administered to normal animals. The possible participation of the RES in the changes evoked by tumor extracts is discussed.—J. Nat. Cancer Inst. 25: 713–718, 1960.

NAKAHARA and Fukuoka (*1*) have reported that an extract of several different human tumors produced a marked diminution in the level of hepatic catalase activity when the extract was administered to normal mice. This observation has been confirmed by a number of investigators, and the progress in the isolation, chemistry, and mode of action of the active material was recently reviewed (*2*).

In addition to its effect on liver catalase, extracts of Walker carcinosarcoma 256 have been shown to lower kidney catalase activity and the concentration of plasma iron in normal recipient rats (*3*). However, when large, repeated, daily injections of tumor extracts were given, they failed to maintain liver and kidney catalase activity and plasma iron at the low concentrations normally found in tumor-bearing animals (*3*).

The release of a toxic substance from tumor tissue would probably be a slow and continuous process. Thus, in the present study, an effort to approach more nearly the conditions that exist in the tumor-bearing animal was made by administering tumor extracts as small, frequent subcutaneous injections. Under these conditions, the catalase enzymes and plasma iron were maintained at a low concentration. This suggested a phenomenon similar to the Dustin effect (*4*) in which under comparable conditions small, repeated doses of drugs stimulated the phagocytic powers of the reticuloendothelial system (RES), while large doses inhibited

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the phagocytosis. Therefore the effects of tumor extracts on the phagocytic ability of the RES were investigated. As a further check on the possible relationship between the RES and iron metabolism, an established stimulator of the RES, Zymosan (*5*) was injected into normal rats and its effects on iron metabolism were determined.

MATERIALS AND METHODS

Tumor extracts were prepared from the non-necrotic portions of Walker carcinosarcoma 256 as the alcohol precipitate, by the method of Nakagawa *et al.* (*6*). Normal tissue preparations were made by skinning a normal rat, removing the intestines, stomach, bladder, and bones, and processing the heart, kidneys, lungs, thymus, liver, spleen, ovaries, adrenals, thyroid, brain, and muscles, by the same method used for preparing tumor extracts.

Female Holtzman rats of the same age, weighing 150 to 180 gm., were used in these experiments. Tumor or normal tissue extracts were suspended in 1 ml. of water and injected in the rats either intraperitoneally or subcutaneously along the back. Zymosan [2] was administered intravenously (*5*).

The methods for liver and kidney catalase activity and plasma iron have been described previously (*7*). The activity of the RES was determined by slight modifications of the method of Biozzi *et al.* (*8*). A suspension of carbon (C11/1431a)[3] was injected at the rate of 16 mg. per 100 gm. body weight into the femoral vein. The rats were kept under light ether anesthesia during the administration of carbon and sampling of the blood. At appropriate intervals blood was collected from the tail vein and a 0.2 ml. sample was pipetted into 5 ml. hemoglobin diluent (1 gm. $NaHCO_3$, 50 mg. KCN, and 200 mg. $K_3Fe(CN)_6$ diluted to 1 liter). The concentration of carbon was determined by spectrophotometer at 540 μ. The blanks were samples of blood obtained prior to the injection of carbon.

RESULTS

The effects which were measured after 5 days of repeated administration of tumor or normal tissue extracts are shown in table 1. Thirty milligrams per day of the tumor-tissue extract given intraperitoneally failed to prevent the return of plasma iron or the catalase activity to normal levels. When the amount of material administered intraperitoneally was decreased to 1 mg. and the frequency of injection to 8 hours, these iron-containing proteins still returned to normal at the end of 5 days. Animals receiving subcutaneous injections of 1 mg. at 2-hour intervals over an 8-hour period, however, maintained a low level of plasma iron and catalase activity

[2] Lot No. 13335S from K and K Laboratories, Jamaica 33, N.Y.
[3] John Hemschel and Co., Inc., 105 East 29th St., New York 16, N.Y.

TABLE 1.—Effect of 5 days of repeated small doses of tumor and normal tissue extracts on the plasma iron, liver catalase, and kidney catalase of normal rats

Preparation used	Route of injection*	Quantity and frequency	Number of rats	Plasma iron ($\mu g.$ %)	Liver catalase†	Kidney catalase†
Sham	ip	0 mg./day	10	274 ± 27‡	5400 ± 200	3600 ± 200
Normal tissue	ip	30 mg./day	6	270 ± 40	5100 ± 200	3200 ± 300
Tumor tissue	ip	30 mg./day	12	236 ± 26	4700 ± 300	3000 ± 200
Tumor tissue	ip	1 mg./8 hr.	6	237 ± 18	5300 ± 300	3200 ± 200
Sham	sc	0 mg. 5 times/day§	12	260 ± 23	5300 ± 200	3400 ± 300
Normal tissue	sc	1 mg. 5 times/day§	12	234 ± 15	5000 ± 200	3100 ± 200
Tumor tissue	sc	1 mg. 5 times/day§	12	111 ± 9‖	3600 ± 200‖	2600 ± 100‖

*Ip = intraperitoneal; sc = subcutaneous.
†Catalase units (7).
‡Mean ± standard error.
§Injections at 8 a.m., 10 a.m., 12 a.m., 2 p.m., and 4 p.m. each day.
‖Significantly different from Sham, $P<0.01$.

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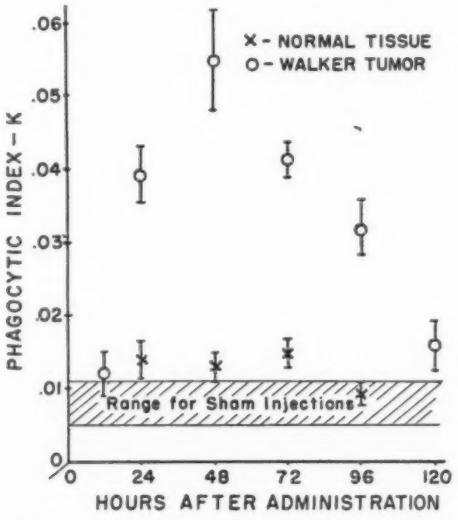
throughout the 5-day period. In a further experiment not shown in table 1, the injections were continued for 10 days and the concentrations of catalases and plasma iron were still maintained at approximately the same levels as those found in tumor-bearing animals.



TEXT-FIGURE 1.—Effect of intraperitoneal injections of 100 mg. of normal or tumor-tissue extracts upon the phagocytic activity of the RES. Extracts were prepared as indicated in "Materials and Methods" and administered in 1 ml. of water. Brackets show the standard deviation of the mean values obtained in 4 to 6 animals.

TABLE 2.—Changes in organ weights and liver and kidney catalase of normal rats 48 hours after administration of 5 mg. of Zymosan

	Sham	Zymosan
Number of rats	15	15
Final body weight (*gm.*)	161 ± 4*	158 ± 4
Liver†	6390 ± 110	7240 ± 230‡
Spleen†	499 ± 14	736 ± 21‡
Thymus†	421 ± 14	346 ± 14‡
Adrenals†	43 ± 3	48 ± 4
Liver§	1140 ± 20	1260 ± 40‡
Spleen§	72 ± 2	104 ± 3‡
Thymus§	59 ± 2	50 ± 2‡
Adrenals§	9 ± 1	10 ± 1
Liver catalase‖	5370 ± 220	3570 ± 170‡
Kidney catalase‖	2880 ± 190	2400 ± 120‡

*Mean ± standard error.
†Mg. wet weight.
‡Significantly different from Sham, $P<0.01$.
§Mg. dry weight/100 gm. body weight.
‖Catalase units (7).

The effect of the tumor-tissue extract on the speed with which carbon particles are phagocytized is illustrated in text-figure 1. The stimulation of the phagocytic activity started shortly after the injection of tumor extract and reached a maximum at 2 days, while the preparations from normal tissues showed only a slight response. Extracts of the Walker tumor, therefore, altered not only the iron metabolism but also the phagocytic activity of the RES when the extracts were injected into normal rats.

The effects of a known stimulator of the RES, Zymosan, on catalase activity and organ weights in normal rats are shown in table 2. The liver and spleen weights of the animals were significantly increased, the thymus weight decreased, but the adrenal weights failed to change. The catalase activity of the liver and kidney was depressed in those animals receiving Zymosan. In addition, it was noted that injections of 5 mg. of Zymosan decreased plasma iron to 20 percent of the normal value in 8 hours.

DISCUSSION

Previous results (*3*) have shown that a single injection of 1 mg. or more of tumor-tissue extract into normal rats decreased plasma iron to approximately 20 percent of its original concentration within 8 hours. Slightly higher doses also produced a marked decrease in liver and kidney catalase activity at 24 to 48 hours. However, 3 to 4 days after a single injection, the plasma iron and catalase activity returned and remained near normal values. If the mode of action of the tumor extracts was through a direct inhibition of synthesis of catalase or plasma iron-binding protein, repeated large doses might be expected to result in further inhibition. However, it was shown previously that, when daily injections of tumor extracts were administered for 7 days, the low levels of plasma iron and catalase activity were not maintained (*3*). This was further confirmed by intraperitoneal injections of tumor extracts (table 1). It has also been demonstrated that tumor extracts have no effect on liver catalase *in vitro* (*1, 9*). It seems likely, therefore, that the effect of these extracts on liver catalase, kidney catalase, and plasma iron was secondary rather than primary. The results of this and previous studies suggest that the RES deserves further study on its possible relationship to these effects (*10*).

During the last few years there have been many studies on the relationship between the metabolism of iron and the function of the RES (*11–13*). These studies showed the effects of the RES upon plasma iron, hemoglobin, and the weights of the liver and spleen. None of them, however, have included measurements of hepatic catalase activity, which has been the primary indicator of the effectiveness of various tumor extracts. The suggestion is made, based on the results of the present experiment, that tumor tissue may contain a higher concentration than normal tissue of a substance(s) which can stimulate the RES. This stimulation may provoke some of the changes observed in normal animals after the injection of these tumor extracts.

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Influence of Pregnancies on the Histogenesis of Mammary Cancer in the RIII/Dm/Se Substrain of Mice [1, 2]

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SUMMARY

An attempt was made to demonstrate how pregnancies affect histogenesis of mammary cancer in RIII mice. The behavior, incidence, number, and relationship of precancerous lesions to the age and reproductive life are compared in virgin, normal-bred, and force-bred mice. The results indicate that pregnancies enhance development of cancer through a promoting action on precancerous lesions. Data are presented which suggest that pregnancies give rise to the nodules both through a subinvolution of the functioning lobule and through a new growth of alveoli—the latter always occurring in the mammary glands of virgins. Both methods operate in the breeders, and the number of nodules in their mammary glands is higher than in the mammary glands of the virgins. Moreover, pregnancies enhance the cancerization of the nodules. Mammary glands of old breeders were not studied because they die of mammary cancer before they reach an advanced age. In the resting mammary tissue of the mammary glands of very old virgins occasional nodules are found which do not become cancers because of the lack of hormonal stimuli that promotes the change from preneoplastic lesions into cancer. Pregnancies provide the quantity of hormones sufficient to promote the cancerization of most nodules. Evidence is accumulating that pregnancies give rise to the precancerous lesion, the plaque, in which growth takes place during the second half of the pregnancy and regression occurs after parturition. The plaques represent the second source of mammary-cancer development in our force-bred mice of the RIII substrain.—J. Nat. Cancer Inst. 25: 719–730, 1960.

OUR KNOWLEDGE of the role of pregnancy in the genesis of mammary cancer is based primarily on the evidence that a higher incidence of mammary cancer develops in multiparous female mice (*1*, *2*) than in those having only one litter (*3*) or in virgins of the same strain.

Among the many contributions to elucidating the problem of the relationship between pregnancy and mammary cancer, two significant observations deserve consideration:

1. Female mice of high mammary-cancer strains have a higher fertility than females of low mammary-cancer strains, and generally mice that never develop mammary cancer have a low fertility (*4*). We compared

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the fertility of force-bred mice that belonged to 3 substrains having a different incidence of spontaneous mammary tumors: 42 RIII/Dm/Se mice (RIII substrain, with 87% mammary cancer in the breeders, at the average age of 327 days), 24 BALB/c mice (C substrain, with 5% mammary cancers in the breeders, at the average age of 600 days), and 18 C57BL mice (C57B substrain, resistant to mammary cancer). After the first mating at 30 days and until 200 days of life, the RIII mice had the highest number of litters (4.38 average), the C57BL the lowest (2.38 average), and the C mice had an intermediate number of litters (3.62 average [*5*]). These observations suggested that a direct relationship exists between fertility of the female mice and tumor incidence. The high fertility also suggests that a hormonal condition exists favoring the development of mammary cancer. This favorable hormonal state is associated with a regular ovarian function in the A strain (*4*).

2. It has also been demonstrated that with an increasing number of litters the incidence of mammary cancer in female mice increases and the tumor age decreases (*2*). When we studied the mammary-cancer development in the RIII substrain, we reported (*6*) that in 43 force-bred females the incidence of mammary cancer increased and the average tumor age decreased in proportion to the increase in the number of pregnancies. It thus appears that the effect of pregnancy on mammary-cancer development is the result of the complex hormonal alterations that accompany pregnancy.

In the present investigation we have attempted to demonstrate how the hormonal stimuli active during pregnancy play a part in the histogenesis of mammary cancer in mice.

Since it is generally agreed that the most important preneoplastic lesions are the hyperplastic alveolar nodule and the "plaque," our observations are especially concerned with these changes in the mammary glands.

The hyperplastic alveolar nodules are composed of grouped alveoli, ranging from few to some hundreds, usually not cystic, and surrounded by a moderately increased layer of connective tissue, without any appreciable evidence of inflammatory reaction (*7*).

The plaques, named from their shape, are more complex lesions and may reach palpable sizes. They are composed of a central portion, or "medulla," of loose, fatty connective tissue, in which normal-looking tubules are scattered, and a more compact "cortex" of radially disposed branching tubules (*8*).

Nodules and plaques are supposed to have the same biological significance in mammary carcinogenesis, in which they represent alternative, not consecutive, steps corresponding to similar biological stages of development.

MATERIALS AND METHODS

The substrain RIII/Dm/Se (RIII) was used in our experiments, and all mice were bred in our laboratory and fed commercial pellets and tap water *ad libitum*. They were grouped as follows:

Virgins: 165. After weaning, 4 or 5 animals were kept in a cage.

Breeders: 157. After weaning, 2 female mice, 30 days of age, were mated with a male of the same litter. At the end of the first suckling, the breeder mice were divided in 2 groups; 133 were allowed unlimited litters and the remaining 24 were allowed only 1 litter. These females were kept alone in a cage after the weaning of the litter.

Force-bred: 43. Two females were kept with 1 male. The young were removed immediately after birth. The females were allowed unlimited litters.

One mouse bred normally and 1 virgin mouse were killed every other day, from the age of 40 days. The force-bred mice were allowed to live until a mammary cancer developed; then 1 was killed every other day.

All the animals were autopsied. All the mammary glands were examined—those on the left side, by the wholemount method, those on the right side, by histological sections.

RESULTS

Mice Bred Normally

In 36 percent of these mice the first parturition occurred before 70 days of age, and in 78 percent within 60 to 100 days. The mice were pregnant for 19 days, and the litters were weaned at 21 days of age.

First group.—Three or 4 days after weaning the first litter, a new pregnancy occurred in 97 percent of the breeders. The mammary glands at this time showed a beginning involution, and within 10 to 12 days the glands had returned to the resting state in all mice, unless another pregnancy had occurred. In these mice, the mammary glands showed the superimposition of the proliferative and secretory modifications.

The second parturition usually occurred at 120 to 130 days, and the weaning of the second litter at approximately 140 to 150 days of age. After the second weaning, 60 percent of the females became pregnant within 10 days, and 40 percent later. Therefore the mammary glands of several mice, killed every other day after the second weaning, were in the resting state and showed only the changes related to the second suckling. It is in these glands that we easily detected the first alveolar hyperplastic nodules, since they appeared in resting tissue.

After this period nodules developed in many animals and the incidence and number increased with the increase in age. The only time the nodules could be found was from the cessation of suckling to the onset of the next pregnancy. Histologically these mammary glands showed involution or beginning involution, except for the nodules. It seems to us that these observations confirm the opinion of many authors who consider the nodules as subinvoluted lobuli of a functioning gland (*9*).

In a previous report we listed the number of nodules in the wholemount sections of the left gland of the second thoracic pair (*10*). From 6 to 38 were observed in a single gland; the number reached the maximum about

the same time that cancer developed, and then began to decrease. We also reported on the distribution of the nodules along the glandular tree, in the same wholemount sections, and the highest number was found in the middle portion.

In the present study, mammary cancer developed in 87 percent of these breeder mice with an average age of 327 days.

Second group.—After the weaning of the first litter, the mammary glands of these mice attained full involution within 10 to 12 days; at this time there were no alveoli and the glands showed a simple ductal structure. The serial study of the mammary glands of these mice was postponed until changes could be anticipated (*11*). At about 100 days we found the first nodules, which were easily detected in the inactive mammary tissue.

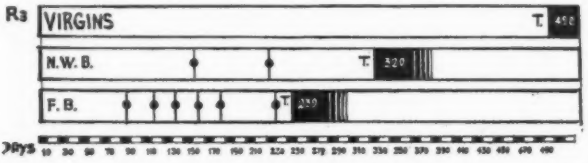
The incidence and the number of nodules increased after this time, with the increasing age of the hosts. In these breeders the nodules represented newly formed clusters of alveoli, which developed in inactive mammary tissue.

Force-Bred Mice

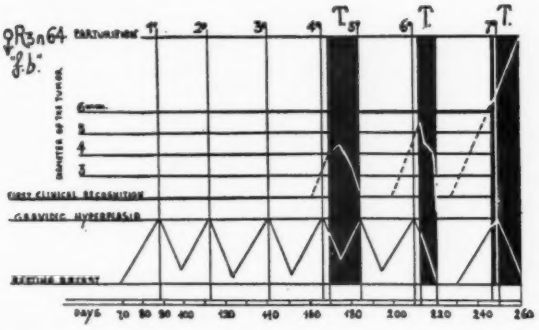
The female mice of this group had an average of 6 litters, and a 99 percent incidence of mammary cancer. The average age when mammary cancer developed in force-bred, normal-bred, and virgin mice is graphically shown in text-figure 1.

During the second half of the second pregnancy, and more frequently in the following pregnancies, the mammary glands of several mice (18%) showed macroscopically recognizable lesions that began to grow but, soon after parturition, decreased and disappeared. During the next pregnancy these lesions again began to grow and become palpable but might again regress after parturition. Finally an irreversible and continuous growth took place as cancer developed. The clinical behavior of the macroscopic mammary lesions is shown in text-figure 2, based on observations made on the RIII female #64.

The histological diagnosis of a plaque, from the observations of Foulds (*12*), is in agreement with the clinical behavior. Corresponding to the phase of the reproductive life in which the mouse was killed, simple, regressive, or progressive plaques could be seen. Near the plaques, the mammary glands of force-bred mice also showed a considerable number of nodules, as reported in table 1 (*13*).



TEXT-FIGURE 1.—Average age, at mammary-tumor(T) development, of RIII virgin, normal-bred, and force-bred mice. The age at tumor development decreases as the number of litters increases $\left(\begin{smallmatrix}|\\\bullet\\|\end{smallmatrix}\right)$.



TEXT-FIGURE 2.—Clinical behavior of a mammary macroscopic lesion and its relationship to pregnancies.

TABLE 1.—Results of histological study of mammary glands of 26 force-bred RIII mice, killed when a mammary cancer was clinically recognizable

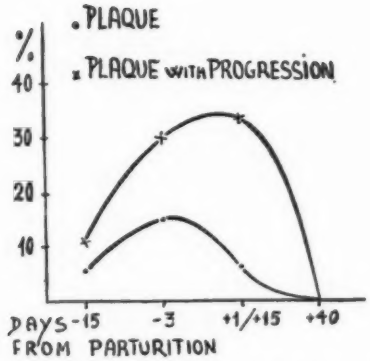
Histological observation	Number of mammary glands	Percent
Resting breast	3	3. 84
Lactating gland	14	17. 94
Nodules	11	14. 10
Plaques	6	7. 69
Plaques with progression	14	17. 93
Cancer	30	38. 46
Total	78	99. 96

To provide a clearer comprehension of the problem of the relationship between the plaque and pregnancy, we have divided the force-bred mice into 4 groups: (1) 5 mice killed during pregnancy, *i.e.*, about 15 days before parturition; (2) 6 mice killed at the end of pregnancy; (3) 8 mice killed 1 to 15 days after the last parturition; (4) 10 mice killed at least 40 days after the last parturition. Text-figure 3 shows that the histological finding of the simple plaque was highest at the end of the pregnancy; progression in the plaques was frequently noted after parturition. Plaques were not found in mice killed many months after the last parturition (text-fig. 4).

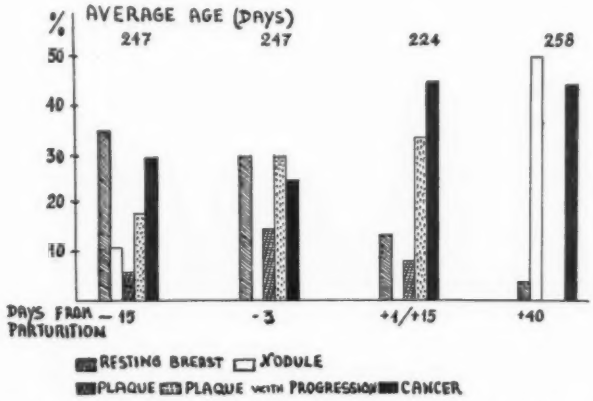
Control Virgins

The average age of the mice, whose mammary glands showed the first nodules, was about 100 days; from this time on, the number of virgins with nodules in their mammary glands and the number of nodules per single gland increased with increasing age, as shown in text-figures 5 and 6 where, for comparison, the incidence and the number of nodules in the mammary glands of the breeders are indicated.

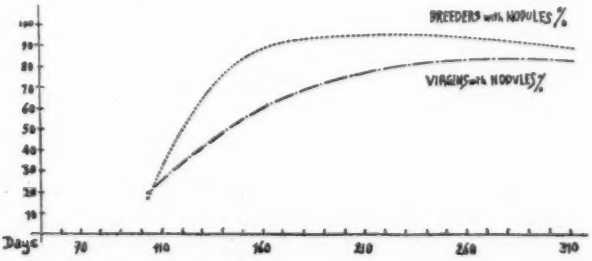
We have never seen a lobuloalveolar differentiation in the mammary glands of the virgins before they were 100 days old, not even in the estrous phase of the cycles.



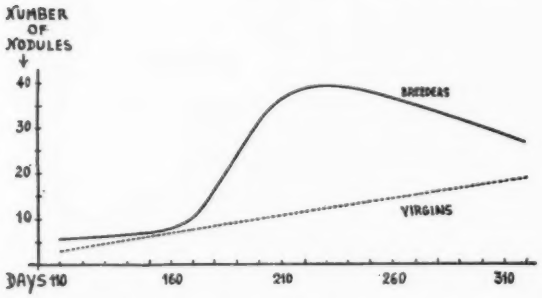
TEXT-FIGURE 3.—Incidence of simple plaque and of plaque with progression in different stages of reproductive life; the highest incidence of simple plaque is found near parturition.



TEXT-FIGURE 4.—Incidence of mammary changes in 4 groups of force-bred mice, approximately the same average age, killed in various periods of reproductive life. The plaque cannot be found in mammary glands of mice killed 40 days or more after the last parturition.



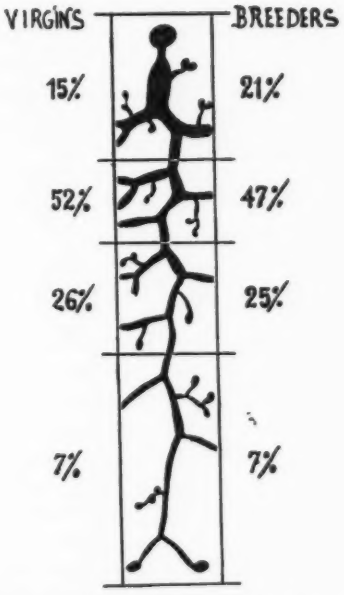
TEXT-FIGURE 5.—Breeders and virgins with nodules; mice were killed every other day, beginning at 30 days of age.



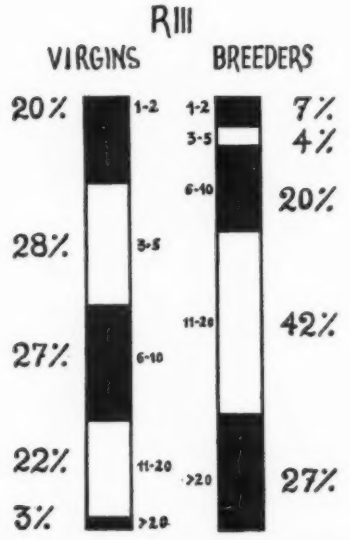
TEXT-FIGURE 6.—Average number of nodules, as revealed by wholemount, in the left mammary gland of the second thoracic pair, in breeders and in virgins.

In the wholemount sections of the left gland of the second thoracic pair, the number and distribution of nodules along the glandular tree, when compared with the results obtained in the study of the mammary glands of the breeders, showed similar results in the distribution of the nodules, but very striking differences in number, which was much lower in the virgins. In these animals we found a minimum of 4 and a maximum of 16 nodules for a single gland (text-figs. 7 and 8).

Mammary cancer developed in 70 percent of virgins at an average age of 380 days.



TEXT-FIGURE 7.—Distribution of nodules, in percent, along the mammary glandular tree (second left thoracic gland). In virgins as in breeders, most nodules are found in the second and third sections of the glandular tree.



TEXT-FIGURE 8.—Percent of tumors. Number of mammary glands with few nodules (from 1–2 to 6–10) is higher in the virgins than in the breeders. Number of mammary glands with many nodules (from 11–20 to beyond 20) is higher in breeders.

After the age at which cancer usually developed, we prolonged the study of the mammary glands of 40 surviving mice, from 400 to 600 days of age, and killed the animals at regular intervals (*14*).

We found a few cancerous lesions in only 3 mice. Generally, the mammary glands were rudimentary and of ductal type. In 70 percent of the animals we noted a considerable number of nodules in otherwise resting glands. This observation shows that the incidence of mammary cancer drops quickly after 400 days, while the incidence of the nodules remains high.

DISCUSSION

The over-all results of this and preceding studies indicate that pregnancy influences mammary carcinogenesis of the RIII mouse through its effect on the precancerous lesions. More cancers at an earlier age develop in breeder mice than in virgins of the same strain, which indicates that pregnancies exercise their influence on the precancerous lesions. Our results emphasize: (1) Pregnancies act on the genesis of the nodules, which arise either as new growths or from subinvolution after pregnancy; (2) pregnancies enhance the cancerization of the nodules; (3) pregnancies give rise to the plaque, the precancerous lesion intimately related to the reproductive life of the host.

Pregnancies act upon the genesis of the nodules in two ways.—(*a*) The alveolar hyperplastic nodules are circumscribed foci of acinar hyperplasia with

secretion, and are similar, histochemically and biochemically (*15*, *16*), to a mammary lobule of pregnancy. The nodules indicate the response of the mammary glandular tree to stimuli of a progesteronic and mammotropic nature. Cortisol stimulates the secretion in the nodules, while in some tumors estradiol gives rise to an increase in the connective tissue in normal and nodule transplants (*17*). The nodule is the more classic precancerous lesion (*18*, *19*). It is reported to differ from prelactating tissue in having large numbers of viruslike particles (*20*). It can be found in the mammary glands of the breeders as well as the virgins of the high mammary-cancer strains, in the mammary glands of the old females of low mammary-cancer strains, and in the mammary glands of male mice after administration of estrogen (*21*).

(*b*) A lobuloalveolar differentiation, *i.e.*, a growth of acini, does not appear during the normal estrous cycle in the mouse (*22*, *23*). Therefore, unless the virgins are given progesterone, any developing nodules must represent a new growth and not merely a lobuloalveolar differentiation developing from hormonal stimulation during pregnancy and persisting after involution of the normal tissue. In the mammary glands of old female mice belonging to low mammary-cancer strains and in the mammary glands of estrogenized males, the nodules develop in the same manner as in virgins and appear as newly formed alveolar clusters.

In the mammary glands of the breeders of the RIII substrain, we have found more nodules than in the glands of the virgins of the same strain. This may be because two mechanisms for the formation of nodules are operating in the breeders: (1) There is a new growth, and (2) the nodules arise through an incomplete involution and regression of a lobule or of a portion of a lobule developed during pregnancy. These two modalities of histogenesis of the nodules have been demonstrated by the results of our study of experimental groups of breeders, and may explain why the mammary glands of the breeders contain more nodules than the mammary glands of the virgins, in spite of the similar morphology, time of development, biological behavior, incidence, and distribution along the glandular tree, in the two groups of mice.

Thus, the probability of the development of a cancer in the mammary glands of the breeder is greater than that in the virgins. If we accept the nodule as a precancerous lesion, we must agree that the greater the number of nodules, the greater the chances for the development of cancer, even though nodules do not become cancers (*14*) and even though the number would be reduced if the suggestion is correct that the cancer developed from a nodule inhibits the cancerization of other nodules (*24*).

Pregnancies enhance the cancerization of the nodule.—While pregnancy gives rise to a greater number of nodules, it also influences the cancerization of the nodules. In 80 percent of the breeders and the high mammary-cancer RIII substrain, cancer will develop and it is difficult to find old breeders because these animals die earlier from mammary cancer. In contrast, a number of virgins belonging to the same strain reached very old age without developing mammary cancer. The tumor incidence, in

the interval from 400 to 600 days, decreased from 70 to 7 percent, while the incidence of the nodules remained high, at about 70 percent. In other words, the nodules in the virgins of this strain do not regress, but apparently lose the power to become cancers. This may be due to the deficiency or the lack of some factor which would change preneoplastic lesions of the breast into tumors. Since pregnancies enhance the cancerization of the nodules, this factor must be recognized in evaluating the hormonal factors provided during pregnancy, because in virgins and breeders of the same strain only the hormonal factor varies, while the genetic and viral factors remain the same.

We may assume from these observations that pregnancy acts upon the genesis and the cancerization of the more frequent precancerous lesion, the alveolar hyperplastic nodule, and in this way exerts its influence on mammary carcinogenesis.

Pregnancies give rise to the plaque.—A second way in which pregnancy influences mammary carcinogenesis in the mouse is its promoting action upon the development of the plaque. In some hybrid mice (*25*), the plaque is the main precancerous lesion from which the tumor derives through focal or diffuse progression. In our material, besides the nodules, the plaques represent a second source of mammary tumors in force-bred mice. The relationship between plaque and pregnancy is especially striking in this material. The plaque is a mammary lesion intimately related to reproduction in female mice. It is a frequent observation especially in the mammary glands of force-bred mice, as in our material, but also in animals bred in a normal way. The plaque is an early step in mammary neoplasia and, since it is of macroscopic size, it is excellent for the study of responsiveness and progression of mammary neoplasia (*12*). This responsiveness can be recognized, at least in the force-bred mice in our study, as a result of hormone dependence, because progression takes place in a plaque after the hormonal stimuli of pregnancy, and the plaque contracts to a small disc or disappears after parturition, as the level of hormones falls.

In our material, the interrelations between plaque and pregnancy are confirmed also by the lack of plaques in the mammary glands of the mice killed at least 40 days after the last parturition and by the regression in the plaques in the mammary glands of animals killed 1 to 15 days after the last parturition.

Since the plaque can disappear (5% in our material) and progression does not take place in all the plaques, it may be suggested that cancerization of the plaques is not inevitable, though we continue to regard it as an important precancerous lesion.

From this study, the conclusion is reached that pregnancy enhances mammary carcinogenesis by promoting the development and the cancerization of the most common precancerous lesion, the nodule, but pregnancy also gives rise to the second precancerous lesion, the plaque, which is intimately related to the reproductive life of the host.

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Effect of Colchicine and Related Substances on the Morphology of Peritoneal Mast Cells [1]

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SUMMARY

Mast cells of the rat peritoneal fluid alter their morphology specifically and characteristically in response to subcutaneous injection of colchicine and some colchicine derivatives; certain other derivatives, metabolic poisons, mast-cell metabolites, and alkaloids do not elicit this response. Similar morphological changes are observed in other types of nondividing cells. Not only are mitotic figures not demonstrable in mast cells after the injection of a variety of antimitotic substances, but the effects observed involve nondividing cells, which demonstrate potent, yet unstressed, paramitotic effects of colchicine at the cellular level. The mast-cell response reflects the effectiveness of the same active colchicines in a number of cytostatic and carcinostatic systems. The relation of the mast-cell response to colchicine is discussed in reference to sol-gel phenomena and contrasted with changes seen in hypophysectomized or in old rats.—J. Nat. Cancer Inst. 25: 731–747, 1960.

MAST CELLS of rat peritoneal fluid are affected characteristically by subcutaneous injection of colchicine (*1–3*) or podophyllotoxin (*3, 4*). In control animals, mast cells are spherical and the nucleus is located centrally; in animals treated with either drug, however, mast cells rapidly become polymorphic and the nucleus becomes peripherally displaced (*1–4*). Preliminary reports that some colchicine and podophyllotoxin congeners are similarly effective have been published (*3, 5*); a more detailed account of such experiments is given here with particular attention to derivatives of colchicine and other pertinent compounds. Specificity of this morphological response will be discussed, and the potency of these drugs as determined by the mast-cell response they elicit will be compared briefly to their potency as mitostatic and tumor-damaging agents.

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MATERIALS AND METHODS

Young adult, male Holtzman rats,[3] weighing 90 to 120 gm. (5–6 weeks old), were used. Food and water were allowed *ad libitum*. Each group was housed separately to avoid drug transfer through coprophagy. Substances to be tested were dissolved in appropriate solvents just before use (table 1) and injected subcutaneously into the dorsal lumbar region. Three hours later, the rats were anesthetized lightly with ether and decapitated. Peritoneal fluid was sampled (1:10 dilution) as previously described (*6*); normal and abnormal mast cells were enumerated in a hemacytometer (entire ruling of 9 mm.^{2} covered) and percentages of abnormal forms calculated. The number of animals tested at each dose varied for each experiment, depending on availability of material and nature of the response (*see* later). Purpurogallin (insoluble in water, ethanol, or propylene glycol) was injected as a suspension at a dose level between 10 and 100 mg. per 100 gm. body weight; enough material was absorbed so that the urine was dark brown 3 hours later at autopsy. Tropolone, prepared according to Drysdale *et al.* (*7*), was dissolved in aqueous sodium bicarbonate. An additional rat was injected intraperitoneally with tropolone dissolved in 1 ml. of physiological saline (*p*H 4.5); 1 ml. of physiological saline, acidified with 0.2 M acetic acid to *p*H 4.5, was used as a control injection. Alloxan-treated animals were deprived of food for 24 hours before the drug was administered and refed 1 hour later; water was available throughout the experiment. A drop of urine obtained directly from the bladder at autopsy was tested for glucosuria with glucose-oxidase paper (Test Tape, Lilly); all 5 animals gave a 4+ reading.

Since nomenclature of the colchicines is not universal (*10*), formulas are given in text-figure 1.

Morphological changes of mast cells evolve in a progressive manner after treatment with colchicine and reflect the dose administered; the percentage of responding cells seems predictable and increases steadily with the dose (*5*). Thus, when only 2 percent of the cells are affected, these are barely abnormal, but when 70 percent are affected, most of them are obviously abnormal. For every active compound at least one group of rats was treated with enough drug, when supplies of the drug or toxicity factors allowed, to elicit this high degree of response; at this response level 3 animals are sufficient for a group and all respond unmistakably.

RESULTS

The data summarized in table 2 show that neither tropolone nor its noncolchicine derivatives—hinokitiol, purpurogallin, and stipitatic acid—were effective. Whereas subcutaneous injection of tropolone did not affect peritoneal mast cells, intraperitoneal injection resulted in extensive

[3] The Holtzman Co., Madison, Wis. The Holtzman rat strain is derived from Sprague-Dawley stock.

TABLE 1.—Substances tested

Substance	Source*	Dose (*mg./100 gm. body weight*)	Solvent	Remarks
A. Tropolones				
1. Colchicine—lots 1150 LMA, 563 LOA, 1248 LRA.	1	0.01, 0.1, 0.2	Saline; water.	Aliquot of lot 1248 LRA was recrystallized twice from ethyl acetate by method of Ashley and Harris (*8*)—all other samples were USP grade
Lot 522630	2			
2. Colchiceine	3	10.0, 100	Saline; water	
3. Deacetylmethyl colchicine	4	0.1, 0.2, 0.4	95% Ethanol: saline = 1 : 1	Colcemid
4. Deacetylmethyl-*N*-isopropyl colchicamide.	3	0.1, 1.0, 10.0	95% Ethanol: water = 1 : 1	
5. Deacetylmethyl-*N*-β hydroxyethyl colchicamide.	3	0.1, 1.0, 10.0	" " " "	
6. Deacetylmethyl-*N*, *N*-dimethyl colchicamide.	3	0.1, 1.0, 10.0	" " " "	
7. Deacetylmethyl-*N*, *N*-pentamethylene colchicamide.	3	10.0	" " " "	
8. Ethylthiocolchicine	3	0.1, 0.5, 1.0, 10.0	" " " "	
9. Trimethylcolchicinic acid methyl ether-D-tartrate.	3	0.1, 1.0, 10.0	" " " "	Dosages as calculated for free base
10. Trimethylcolchicinic acid	5	1.0, 10.0	" " " "	Slight heating used to dissolve NCI #1893
11. Colchinol	5	1.0, 10.0	50% Propylene glycol: ethanol = 1 : 1	NCI #1896
12. *N*-acetyl colchinol	5	0.1, 1.0, 2.0	50% Propylene glycol	NCI #1894
13. *N*-acetyl colchinol methyl ether	5	0.1, 0.5, 1.0	" " "	NCI #1895
14. Purpurogallin (1, 2, 3-trihydroxy benztropolone)	6	Between 10 and 100	Suspended in saline	Lot 5337
15. Hinokitiol (4-isopropyl tropolone)	6	100	50% Ethanol	Lot 9193
16. Stipitatic acid (3-hydroxy-5-carboxy-tropolone)	7	40	50% Ethanol	Aqueous NaOH used first until dissolved
17. Tropolone		5.0, 10.0, 25.0, 100 (subcutaneous), 7.7 (intraperitoneal)	Aqueous $NaHCO_3$	

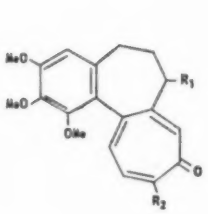
See footnotes at end of table.

TABLE 1.—Substances tested—Continued

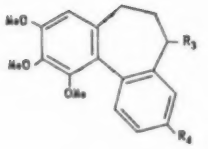
Substance	Source*	Dose (*mg./100 gm. body weight*)	Solvent	Remarks
B. Antimitotics and growth inhibitors				
18. Urethan (ethyl carbamate)	8	100, 200, 500	Saline (0.9% NaCl)	Lot 12672.—Anesthesia induced at higher doses;
19. 4-Amino-*N*-methylfolic acid (amethopterin)	9	0.3	Aqueous NaOH	Methotrexate (lot 7-5173)
20. 2, 4-Diamino-5-(3′, 4′-dichlorophenyl)-6-ethyl pyrimidine	10	25.0	Aqueous acetic acid	Burroughs Wellcome & Co., cpd 50-276 (lot BM-214-2)
21. 2, 6-Diaminopurine	10	10.0	NaOH in saline	Burroughs Wellcome & Co., cpd 48-14a (lot BP-138)
22. 8-Azaguanine	10	10.0	" "	Burroughs Wellcome & Co., cpd 47-28b (lot XB 45b)
23. 6-Mercaptopurine	10	10.0, 30.0	" "	Burroughs Wellcome & Co., cpd 51-30 (lot AM 33537)
24. Sodium cacodylate	8	150	Distilled water	Lot 311091
25. Holothurin	11	0.8, 8.0	Saline	Lot 4/17/57, Toscas Key, Bimini
C. Drugs affecting nervous system, etc.				
26. Atropine sulfate	12	70	Distilled water	Lot CVP—USP grade
27. Pilocarpine nitrate	13	30	Distilled water	Lot 3382—USP grade
28. Pentobarbital	14	0.6		Veterinary Nembutal sodium; anesthesia induced for entire 3 hrs. of expt.
29. *d*-Tubocurarine	14	0.025	Distilled water	
D. Mast-cell metabolites				
30. L-Histidine	15	10.0		CfP grade (lot 150931)
31. Histamine base	15	100	Aqueous orthophosphoric acid	CfP grade
32. Heparin	16	10.0, 20.0 (subcutaneous or intraperitoneal)	Saline	Lot 213—1 mg. equivalent to not less than 60 Toronto units

E. Mast cell-damaging agents and antimetabolites				
33. Compound 48/80	17	10.0	Saline	Burroughs Wellcome & Co., cpd 48-80d
34. *Pseudomonas* polysaccharide	18	0.005, 0.01		Piromen (lot P 92)
35. Aminoguanidine bicarbonate	8	1.0	Saline	
36. Reserpine base	4	0.5	Suspension in saline	No sedation elicited
Reserpine phosphate (lyophilized)	4	6.4–7.6	Distilled water	Animals moribund at 3 hrs.—Serpasil phosphate
F. Metabolic poisons				
37. Fluoride		20.0	Distilled water	Sodium salt used
38. Iodoacetic acid	19	5.0	Distilled water	Sodium salt used, NaI-free
39. 2, 4-Dinitrophenol	2	1.5	Ethanol: water = 1 : 1	Lot 752700
40. Arsenite	12	1.0	Distilled water	AR grade (lot WCKSA), sodium salt
41. Azide	13	3.0	Distilled water	Sodium salt used
42. Malonate	8	250	Distilled water	Sodium salt used—dose not corrected for water of crystallization
43. *p*-Chloromercuribenzoate	20	7.0	Distilled water	Lot 105–106
44. Phlorhizin	13	100	Aqueous sodium bicarbonate	Lot 9522
45. Alloxan	19	20	McIlvaine buffer, *p*H 4.0	(*see* reference *9*)
46. Mersalyl acid	21	2.5, 5.0	NaOH in saline	Salirgan—final *p*H = 7

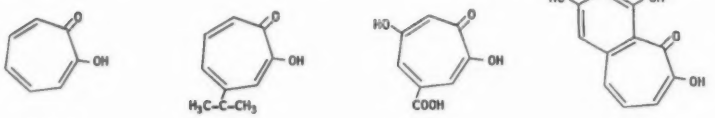
*1) Courtesy Messrs. G. Motoasca and P. W. Riebling, S. B. Penick and Co., New York, N.Y. 2) Fisher Scientific Co., New York, N.Y. 3) These materials, prepared by Drs. Wettstein and Anner, Ciba Ltd., Basel, Switzerland, were obtained through the courtesy of Dr. R. Gaunt, Ciba Pharmaceutical Products, Inc., Summit, N.J. 4) Colcemid and reserpine compounds courtesy Drs. R. Gaunt and J. Cooper, Ciba Pharmaceutical Products, Inc., Summit, N.J. 5) Courtesy Dr. J. L. Hartwell, Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda 14, Md. 6) K and K Laboratories, Inc., Jamaica 33, N.Y. 7) Courtesy Dr. S. Tannenbaum, Dept. of Biochemistry, Columbia University, New York, N.Y. 8) Matheson-Coleman and Bell, East Rutherford, N.J. 9) Courtesy Dr. T. H. Jukes, Lederle Laboratories, Pearl River, N.Y. 10) Courtesy Dr. S. Bieber, Burroughs Wellcome & Co., Tuckahoe 7, N.Y. 11) Courtesy Dr. R. F. Nigrelli, New York Aquarium, New York Zool. Soc., N.Y. 12) Mallinckrodt Chemical Works, St. Louis, Mo. 13) Nutritional Biochemicals Corp., Cleveland 28, Ohio. 14) Abbott Laboratories, North Chicago, Ill. 15) California Corporation for Biochemical Research, Los Angeles 63, Calif. 16) Hynson, Westcott, and Dunning, Baltimore, Md. 17) Courtesy Dr. E. J. De Beer, Burroughs Wellcome & Co., Tuckahoe 7, N.Y. 18) Courtesy Travenol Laboratories, Inc., Morton Grove, Ill. 19) Distillation Products Industries, Eastman Organic Chemicals, Rochester 3, N.Y. 20) Sigma Chemical Co., St. Louis, Mo. 21) Winthrop Laboratories, New York, N.Y. Sample obtained through courtesy of Dr. A. M. Zimmerman, Dept. Pharmacol., Downstate Medical College, New York, N.Y.



	R_1	R_2
Colchicine	$NHCOCH_3$	OCH_3
Colchicoine	$NHCOCH_3$	OH
Deacetylmethyl colchicine	$NHCH_3$	OCH_3
Deacetylmethyl N-isopropyl colchicamide	$NHCH_3$	$NHCH(CH_3)_2$
Deacetylmethyl N-β-hydroxyethyl colchicamide	$NHCH_3$	$NHCH_2CH_2OH$
Deacetylmethyl N,N-dimethyl colchicamide	$NHCH_3$	$N(CH_3)_2$
Deacetylmethyl N,N-pentamethylene colchicamide	$NHCH_3$	$N(CH_2)_5$
Ethylthiocolchicine	$NHCOCH_3$	SC_2H_5
Trimethylcolchicinic acid	NH_2	OH
Trimethylcolchicinic acid methyl ether	NH_2	OCH_3



	R_3	R_4
Colchinol	NH_2	OH
N-acetyl colchinol	$NHCOCH_3$	OH
N-acetyl colchinol methyl ether	$NHCOCH_3$	OCH_3



TEXT-FIGURE 1.—Formulas of colchicine derivatives and related substances tested. Me represents a methyl group, CH_3.

damage to the local mast-cell population, as evidenced by large numbers of free granules and widespread uptake of mast-cell fragments by phagocytes. None of the morphological alterations typical of colchicine were noted. Intraperitoneal injection of saline acidified to *p*H 4.5 (control) did not affect mast cells. Some of the colchicine congeners tested proved effective in altering mast-cell morphology, and variations in potency measured by the mast-cell response were evident. Compounds 18 to 46 (*cf.* table 1) did not affect mast-cell morphology regardless of dose level and, consequently, were omitted from table 2. A number of colchicine derivatives likewise proved inactive. For active compounds, as for colchicine, the percentage of abnormal mast cells in an affected population reflected the dose administered. In table 2 any *consistent* response is expressed as positive (+), whether marked or barely evident; more subtle observations are not shown in the table. For instance, *N*-acetyl colchinol was somewhat less active than its methyl ether, the former yielding an average of 72 percent affected mast cells at the 1.0 mg. dose as compared with 95 percent for the same dose of methyl ether. With active colchicine derivatives, whenever mast cells became abnormally shaped, many monocytoid cells and eosinophilic leukocytes of peritoneal fluid also exhibited similar alterations (figs. 1 through 15). Alteration of mast cells (and other cells) was in no case ascribable to the solvents used since it proved possible to lower the dose of all active congeners to a point where no response was elicited. Compounds which did not elicit, in mast cells, the particular morphological endpoint considered here, generally did not

TABLE 2.—Activity in mast-cell system

Compound	Dose (*mg./100 gm. body weight*)										
	100	40	25	10	2	1.0	0.5	0.4	0.2	0.1	0.01
1. Colchicine, lot 522630										+	−
lot 1150 LMA										+	−
lot 563 LOA									+	+	−
2×recryst.										+	−
2. Colchiceine	−			−							
3. Deacetylmethyl colchicine								+	+	−	
4. Deacetylmethyl-*N*-isopropyl colchicamide				+		−				−	
5. Deacetylmethyl-*N*-β hydroxyethyl colchicamide				+		−				−	
6. Deacetylmethyl-*N,N*-dimethyl colchicamide				+		+				−	
7. Deacetylmethyl-*N,N*-pentamethylene colchicamide				−							
8. Ethylthiocolchicine				+		+	+			−	
9. Trimethylcolchicinic acid methyl ether				+		+				−	
10. Trimethylcolchicinic acid				−		−					
11. Colchinol				−		−					
12. *N*-acetyl colchinol					+	+				−	
13. *N*-acetyl colchinol methyl ether						+				−	
14. Purpurogallin				−							
15. Hinokitiol	−										
16. Stipitatic acid		−					−				
17. Tropolone			−	−							

have any other visible effect on these cells; only compound 48/80 and alloxan appeared to cause a slight departure from normal, but this was in no way similar to the characteristic involvement of nucleus and cytoplasm observed in response to colchicine-like drugs. Under no circumstance was a mast cell seen in mitosis, regardless of treatment.

DISCUSSION

Shape of free-floating peritoneal mast cells.—The morphological response of mast cells to colchicine has been described in detail elsewhere (*1–3*) but, in view of the adjustments in shape described for mast cells in various physiological or experimental conditions, they are sketched in text-figure 2. Free-floating mast cells normally are seen in peritoneal lymph; in young rats, they are spherical and their nucleus is centrally located. In response to injection of colchicine, these same mast cells become nodular—the cytoplasm appears segmented, and the nucleus is invariably located at one pole of the cytoplasmic mass, peripherally displaced to such an extent that a good part of its surface appears naked of cytoplasm. This type of deformation must be differentiated from the less-marked departure from the spheroid condition that occurs in old animals (*11*) or follows hypophysectomy (*12*). It should be noted that only over-all shape of the cell and relative position of the nucleus are involved; the characteristic mast-cell granules are present in all cases and are only passively affected to the extent that they conform to the plasticity of the cytoplasmic mass.

Specificity of response to colchicine.—The mast-cell response to colchicine is remarkably specific. To date, only colchicine and some of its derivatives as well as podophyllotoxin and some of its congeners (*3*, *4*) have proved effective in altering mast-cell morphology. These two families of drugs inhibit mitosis through interference with spindle formation.

Table 2 shows that whereas some small structural modification of colchicine may result in virtual abolition of activity, *i.e.*, colchiceine,

TEXT-FIGURE 2.—Morphology of mast cells in rat peritoneal fluid under various conditions seen in the hemacytometer: *A*. Normal, spherical shape seen almost exclusively in younger animals. *Note* centrally located nucleus. *B*. Segmented cytoplasm and peripheral location of nucleus (*n*) characteristic of colchicine response (at minimum effective dose, as defined in this paper). *C*. Nonspherical mast cell from hypophysectomized rat. *D*. Nonspherical mast cell from older rat. In *A*, *C*, and *D* the nucleus is only partially overlaid with granules and appears as a clear area.

activity nevertheless is retained even after relatively major modification, such as conversion of the tropolone ring C of colchicine to the 6-carbon ring-C system of *N*-acetyl colchinol and its methyl ether. A detailed discussion of potency as a function of molecular structure will not be undertaken here because present findings with the mast-cell response parallel in every respect those based on tumor damage *in vivo* (*13–17*) and regenerating rat liver (*18, 19*), as partially reviewed by Eigsti and Dustin (*10*). It must be emphasized that a wide variety of compounds proved ineffective in the mast-cell system despite the fact that most of them proved effective in one or more test systems described in the literature. This is true of cacodylate (*20*), which affects the mitotic apparatus and leads to c-mitosis (*21*), and of the alkaloids chelidonine and protopine (*22*) from *Chelidonium majus*, whose sap has been used to treat warts in much the same way as colchicine and podophyllin. The noncolchicine compounds screened were chosen for their reported antimitotic or antitumor activity, their colchicine-like pharmacological properties, their role in mast-cell metabolism, or their ability to poison biochemical pathways. Space considerations preclude further discussion of these points.

Because of the two 7-membered rings of colchicine, compounds other than colchicines that contain a cycloheptanone nucleus, and simple tropolone derivatives have been investigated in various laboratories for their effect on mitosis. Ascites cells are not affected by such compounds, including hinokitiol and tropolone itself (*23*); only one tropolone derivative, *o*-bromo-*p*-aminotropolone, was reported to cause c-mitosis in ascites tumor cells (*24*). In tissue culture, tropolone is ineffective in blocking mitosis (*25*) and may actually antagonize the action of colchicine (*26, 27*) although there is no general agreement on this point (*25*).

In all cases, doses administered in the present experiments were close to, or above, reported LD50 levels; 3-hour survival of an animal was the only prerequisite. Since many mitotic poisons have proved ineffective in the present experiments, it must be concluded that all the test systems mentioned reveal activity for a much greater variety of the substances we have tested than was actually the case for eliciting morphological alterations in mast cells. For active colchicine derivatives, the threshold dose is fairly constant for all test systems, except for the leukocyte migration system (*28*) which is much less sensitive.

It is unlikely that well-differentiated mast cells seen in normal connective tissues of the rat can undergo mitosis (*3*). The fact that mitosis has been reported for what appears to be "mast cells" in tissue culture or in mastocytomas must await further critical examination and a more specific definition of the mast cell. It is significant that, among thousands of mast cells critically examined in this study, not a single cell was observed in a stage that could unequivocally be referred to as mitosis, despite the fact that a variety of mitostatic compounds were investigated. A binucleate mast cell was seldom observed, and it was always spherical and without evidence of incipient furrowing. On this basis, it is particularly interesting that antimitotic agents, such as colchicine (*1, 2*) and its deriva-

tives (*3*) should effect a specific morphological response in this type of cell. The mast cell is fragile and cytolizes or degranulates following mild trauma caused by a large number of chemical and physical agents; this common response, however, is not comparable in any way with that elicited by colchicines, and therefore the action of the latter should not be considered evidence of nonspecific toxicity. Analysis of morphological alterations after administration of colchicine suggests that a single process is involved (*5*), and that it is intimately linked to the sol-gel status of the cell. Since the shape of the mast cell after treatment with colchicine differs from that seen under other conditions (text-fig. 2), it is further suggested that a highly specific lesion is indicated from observations reported in this paper.

Evaluation of drug activity.—Derivatives of colchicine have been studied extensively in the hope of finding compounds having greater carcinostatic potency and a reduced "general" toxicity. Various test systems described in the literature have provided standard means of evaluating such compounds. Interference with mitosis is the mechanism underlying these systems, with the exception of a leukocyte motility test (*28*) and, perhaps, *in vivo* tumor-damage assays. Indeed, it has been suggested by Ludford (*29*) that tumor-damaging activity dependent on hemorrhage is due to interference with the developing vascular bed of the growing neoplasm and, in support of this hypothesis, he quotes Dustin and Chodkowski's data (*30*) as evidence that newly formed capillaries are particularly sensitive to mitotic poisoning by colchicine. The hemorrhagic effect, it is held, resulted from inhibited mitoses in capillary endothelium cells despite Ludford's own emphasis of findings that to produce hemorrhage in tumors ten times more colchicine is needed than to produce mitotic inhibition. Dustin and Chodkowski's report (*30*) claimed no greater sensitivity for capillary endothelium and, indeed, a single dose was used throughout their experiments. In their wound-healing experiments, capillary vessels were among the *first* to develop and hence they stated that blocked mitoses could be revealed earliest by administration of colchicine, particularly in the "endothelium des capillaires." Ludford's hypothesis that hemorrhagic tumors result from altered capillaries remains tenable, but contrary to his interpretation, it seems possible that this results from some extramitotic effect of colchicine rather than from inhibition of mitotic activity *per se* in capillary endothelium. Vasoconstriction also has been observed after injection of colchicine (*31*). The leukocyte motility test clearly depends on some factor other than mitosis since the buffy-coat leukocytes used are well differentiated and incapable of cell division. In view of these and other arguments, the mast-cell system used in the present experiments is of particular interest in elucidating the mechanism of action of colchicine.

Because compounds can be screened readily and rapidly with the mast-cell system, which requires few animals and small amounts of material, studies correlating drug activity as measured by various test systems are of particular practical interest. Correlative studies suggest good corre-

spondence between the mast-cell system and those assays that rely on blocking mitosis. The only system previously discussed that is clearly independent of mitotic activity, *i.e.*, leukopedesis, does not correlate well with the mast-cell system; in this *in vitro* assay absorption and excretion rates, deactivation or other metabolic alterations by specific organ systems, and homeostatic mechanisms cannot enter into play as they might when the drug is administered *in vivo*. Yet this applies to fibroblasts in tissue culture in which good parallelism was demonstrated. Leukocyte migration is much less sensitive to inhibition by colchicine than is mitosis (*28*), thus further dissociating this system from the others; no correlation appears to exist between the percentage of inhibition of leukocyte migration and MED for mitosis of tissue-culture fibroblasts.

A high degree of specificity exists with respect to agents eliciting the mast-cell response, but morphological alteration in response to colchicines is emphatically not unique for mast cells. Many other cells in peritoneal fluid react similarly to colchicine, including mononuclears and eosinophils (figs. 1 through 15); in these cells, the nucleus does not project markedly out of the cytoplasmic mass, as in mast cells, but definite polarization ensues—the cell elongates, the nucleus is near one pole, and the cytoplasm becomes irregularly nodular. This cytoplasmic reaction, clearly described by earlier investigators, was overshadowed by striking effects on the mitotic apparatus proper. Ludford showed (fig. 5 in ref. *32*) a cell in tissue culture with arrested c-mitosis and a "vacuolated cytoplasm . . . being divided into two parts." Another cell (also in mitosis) treated with colchicine was ". . . in the act of budding off the greater part of its cytoplasm . . . ." His supporting photomicrograph (plate 17 in ref. *32*) is identical with many we obtained after injection of colchicine for *resting* peritoneal fluid cells other than mast cells. Recently, similar cytoplasmic effects of colchicine congeners and of xanthopterin were described for bone marrow cells in tissue culture (*33*), and excellent photomicrographs show all such affected cells arrested in mitosis. These cytoplasmic changes were emphasized, "Unabhangig von der gestorten Kernteilung kann die Cytoplasmateilung dieser Zellen bis zu ihrem Ende ablaufen." These findings are thus interpreted to mean nuclear but not cytoplasmic division is blocked, as evidenced by "furrowing": "Bei vielen dieser Zellen sind alle Übergänge von einer beginnenden Einschnürung bis zur völlingen Durchsnürung des Cytoplasmas *in zwei* [italics added] annähernd gleich grosse Teile zu erkennen, so dass der eine Plasmaleib die gesamte Chromatinmasse enthält, während der andere davon frei bleibt." Accompanying photomicrographs (*33*) show more than one "cleavage furrow" per cell, but this circumstance escaped special comment. Present findings with nondividing cells of peritoneal fluid (mast cells, eosinophils) and with resting or interphase monocytic cells suggest that morphological variants observed in Ludford's and Albrecht's experiments were not restricted to cells undergoing mitosis and that such changes do not actually represent an abnormal progression of the mitotic process.

It seems more plausible that colchicine directly or indirectly affects some

contractile or expansile proteins perhaps present in most cells. Protein of spindle fibers is apparently affected by colchicine in live cells, but other morphotic proteins are present (*34–37*) and may also be involved, depending on types of cell and environmental conditions. Colchicine did not affect the ability of adenosinetriphosphate to cause contraction of extracted fibroblasts (*35*) but it could do so indirectly *in vivo*. A similar hypothesis was advanced by Brachet (*38*) and Lettré (*22*). Further research in this area is necessary before attempting to relate such effects to the peculiar cytoplasmic response of live cells after exposure to colchicine.

Mast cells may exhibit shifts in their degree of solation under physiological conditions. Thus, variations in their morphology with aging (*11*) can be ascribed to sol-gel alterations (*39–41*). Morphological changes in mast cells of hypophysectomized rats (*12*), similar to those seen in aging animals (*11*), may also reflect a shift in sol-gel equilibrium. As discussed (text-fig. 2), pleomorphism of peritoneal-fluid mast cells in hypophysectomized and in old rats differs markedly from that after treatment with colchicine (*3*), but response to colchicine also involves sol-gel changes as demonstrated with high hydrostastic pressures (*42*). A normally well-developed contractile component might contribute to the ability of mast cells to appose themselves intimately to connective-tissue fibers—so intimately that they were thought to display "secretion channels" (*43*) until carefully reinvestigated by Webb (*44*).

A few colchicine derivatives are active in our mast-cell system, probably because of similarities in molecular configuration. Conversion to colchicine (most active of them all) or to a common intermediate seems unlikely because of the diversity of metabolic alterations necessary to equate them and because podophyllotoxin, whose molecular configuration differs appreciably from that of the colchicines, is similarly active (*3*). Mast-cell alterations evident within 1 hour or less would necessitate rapid conversion to a hypothetical common intermediate. Furthermore, 80 to 100 percent of an injected dose of colchicine can still be recovered unchanged after 6 hours (*45*) and only 32 percent of an injection of colchicine is accountable, in the rat, as $C^{14}O_2$ over 24 hours after treatment, 62 percent being traceable to urine and feces as C^{14} of unspecified constituents (*46*). Colchicines act directly in tissue culture and *in vivo* where topical application blocks mitosis locally, but not at distant sites (*47*), so that any common intermediate would have to be formed within the affected cells.

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PLATE

Morphological alterations of peritoneal cells in response to colchicine. All photomicrographs on this plate are from air-dried smears of rat peritoneal fluid stained with hematoxylin-chromotrope 2R-toluidine blue after methanol fixation. Variation in size of cells is dependent on area of smear.

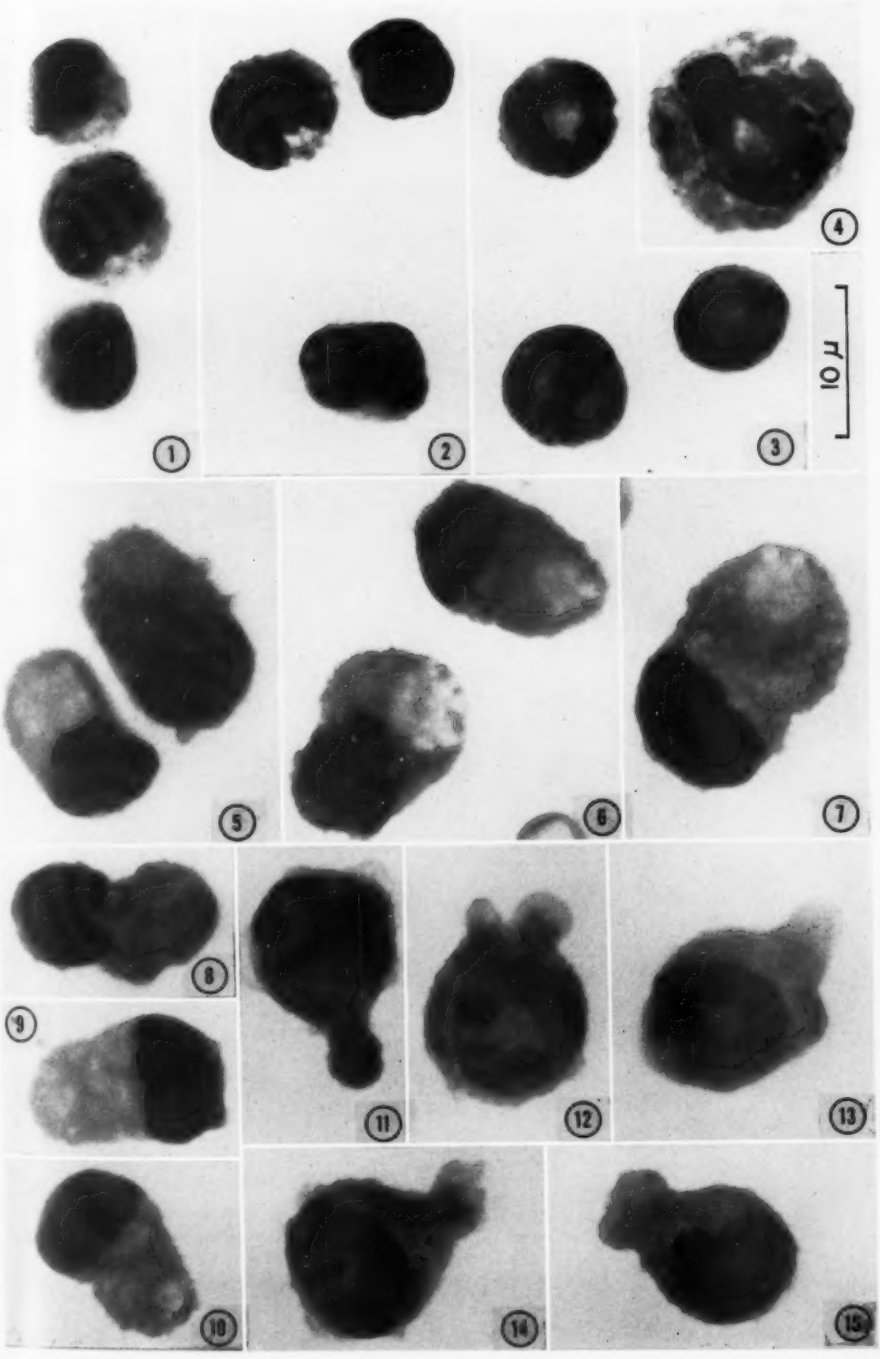
PLATE 105

FIGURES 1 and 2.—Normal mononuclears—uninjected control. *Note* cells are approximately spherical.

FIGURES 3 and 4.—Normal eosinophils—uninjected control. Doughnut nucleus is characteristic of the mature rat eosinophil. *Note* cells are spherical, granules evenly distributed throughout cytoplasm.

FIGURES 5 through 10.—Affected mononuclears—colchicine-treated rat. *Note* cells are definitely elongated and nucleus positioned at one pole. Cytoplasm is somewhat nodular especially in 8. Prominent vacuole is generally present in affected mononuclears.

FIGURES 11 through 15.—Affected eosinophils—colchicine-treated rat. *Note* cytoplasmic blebbing and lack of granules at tip of blebs. Prominent vacuole is only occasionally found in eosinophils.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Potential Oncogenic Properties of High Polymers in Mice [1]

WILLIAM L. KYDD *and* LEO M. SREEBNY, *Department of Prosthodontics, and Department of Oral Pathology, School of Dentistry, University of Washington, Seattle, Washington*

SUMMARY

This experiment was designed to determine the oncogenic potential of a polymerized epoxy resin and an autopolymerizing methylmethacrylate. Two hundred, 6-week-old Harlan stock albino Swiss mice were used. One of the following 4 resins was subcutaneously implanted into the lateral abdominal wall of 50 animals: (1) epoxy resin (Epon 828 diethylenetriamine), (2) autopolymerizing methylmethacrylate (Caulk, NuWeld, Batch #8666), embedded 1 hour after processing, (3) heat-cured methylmethacrylate (Luxene), and (4) cellophane (5½ H. S. Visking sausage casing). The animals were observed daily until the incisions had healed, and then weekly for the presence of subcutaneous tumors. Under the conditions of this experiment no tumors were produced with any of the agents tested.—J. Nat. Cancer Inst. 25: 749–751, 1960.

RECENT EXPERIMENTS by Oppenheimer *et al.* (*1*) and Laskin *et al.* (*2*) have shown that heat-cured methylmethacrylate and cellophane are oncogenic in mice. Hine *et al.* (*3*) showed that uncured aliphatic epoxy resins of low molecular weight were oncogenic in mice. An uncured aromatic resin, however, did not induce tumors. This experiment was designed to determine the oncogenic potential of a polymerized epoxy resin and an autopolymerizing methylmethacrylate.

METHODS AND MATERIALS

Two hundred, 6-week-old Harlan stock albino Swiss mice [2] were used in this experiment. One of the following 4 resins was implanted subcutaneously into the lateral abdominal wall of 50 animals: (1) an aromatic epoxy resin (Epon 828, diethylenetriamine), (2) autopolymerizing methylmethacrylate (Caulk, NuWeld, Batch #8666), which was embedded 1 hour after processing, (3) heat-cured methylmethacrylate (Luxene), and (4) cellophane (5½ H. S. Visking sausage casing).

[1] Received for publication March 31, 1960.

[2] These were purchased from Harlan Small Animal Industries, P.O. Box 134, Cumberland, Ind.

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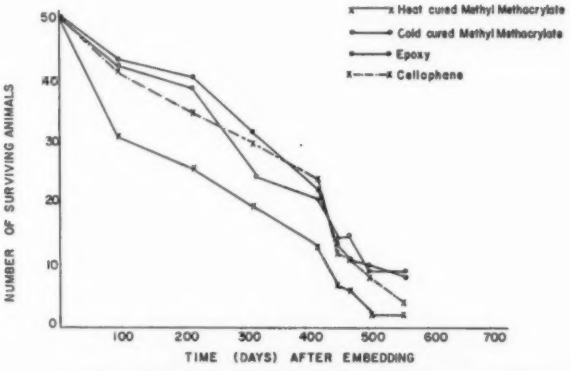
The resins were processed to a thickness of 0.010 to 0.015 inches and then hand-sanded with silicon carbide sandpaper (240 to 400 grit) to remove all mold release agents or separating materials. After sanding, the specimens were 0.010 to 0.008 inches thick. The cellophane was preprocessed to a thickness of 0.004 inches and was used in this form. All specimens were 1 × 1 cm. with slightly rounded corners. They were sterilized in 1:1000 benzalkonium chloride (Zephiran) for 20 minutes. The animals were observed daily until the incisions had healed, and then weekly for the presence of subcutaneous tumors.

RESULTS AND DISCUSSION

No tumors were observed for 420 days. Within this period, however, 119 mice died from causes apparently unrelated to the experimental procedures (text-fig. 1). One tumor was detected at 420 days in a mouse that had been implanted with heat-cured methylmethacrylate. This tumor was located 1 cm. from the site of the implant. Microscopic examination of the lesion showed that it was a mammary-gland carcinoma. The experiment was terminated 575 days postoperatively at which time 27 animals were still living. Autopsy findings showed that 23 of these animals retained their embedded resins. However, no additional tumors were observed.



RELATION OF SURVIVING ANIMALS WITH TIME

TEXT-FIGURE 1.

These results differ from those of Laskin *et al.* (*2*) who observed 5 sarcomas in 50 animals embedded with methylmethacrylate (20 of these 50 animals survived after 257 days) and 1 sarcoma in a similar number of mice implanted with cellophane. This experiment was closely patterned after that of Laskin *et al.*, the only difference being the method of removing mold release agents. These might enhance the capacity of certain resins to produce sarcomas.

Oppenheimer *et al.* (*4*) found that polymer films of numerous plastics will produce sarcomas in rats. Fibers, powders, or perforated implants of the same plastics, however, led to a marked reduction in the incidence of these tumors (*5*).

Although the conditions of the experiments of Hine *et al.* (*3*) are quite unrelated to ours, we lend support to their views that the aromatic epoxy resins are not oncogenic in mice. Under the conditions of our experiment, no tumors were produced with any of the agents tested.

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Viruslike Particles in Precancerous Hyperplastic Mammary Tissues of C3H and C3Hf Mice[1, 2]

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SUMMARY

Viruslike particles in C3H/Crgl mice, seen with the electron microscope, are abundant in hyperplastic mammary tissues that are not neoplastic but known to be preneoplastic. This includes tissue from hyperplastic alveolar nodules and from hyperactive outgrowths of nodules transplanted into gland-free mammary fat pads. Similar hyperplastic tissues from four different C3H sublines presumably lacking the mammary-tumor virus contain abundant viruslike particles indistinguishable from those in C3H/Crgl tissues. Outgrowths from normal transplants, normal hyperplasias (prelactating lobules) of pregnancy, and "noduloids" induced by the administration of estrogen or cortisol typically contain only rare, scattered particles. The data suggest that the viruslike particles are primarily associated with the precancerous mammary hyperplasias; their relationships to inception of tumor and to the mammary-tumor virus remain unknown.—J. Nat. Cancer Inst. 25: 753–777, 1960.

ELECTRON-MICROSCOPE studies of viruslike particles in mammary carcinomas of mice, reported by many laboratories, provide a general conception of their appearance and distribution, though there are some discrepancies. Two kinds of particles are commonly seen. A ring-shaped profile 65 to 80 mμ in diameter, called the A particle by Bernhard (*1*), appears only in the cytoplasm. A larger form, 100 to 120 mμ in diameter and usually extracellular, with an outer double membrane and a central or eccentric dense nucleoid, is designated the B particle by Bernhard. A particles may be scattered in the cytoplasm or may occur in aggregates, frequently in or near the Golgi area and occasionally associated with membrane-bounded vacuoles (*2*). They are also often seen in or below microvilli at the margin of the cell. B particles, usually found in the lumina, are occasionally seen in vacuoles in the cytoplasm. A movement of A particles to the cell margin and their extrusion from

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microvilli to form B particles has been postulated [*see* Bernhard (*1*) for further comments and citations]. Lasfargues *et al.* (*3*) illustrate thickenings of the cell membrane in the form of crescents, horseshoes, and incomplete dense spheres projecting into intercellular spaces, and propose a sequence of steps in virus formation at this site. They suggest that intracytoplasmic A particles are not part of the same series.

The studies contributing to this conception have been made with tumors from many different strains of mice known to harbor the mammary-tumor virus. In spite of an accumulation of evidence (*1, 4, 5*) unequivocal proof of the identification of the particles seen in the electron microscope with the biologically active virus is lacking. Some tumors from animals presumed to be virus-free have been shown to contain similar particles (*6–8*). Furthermore, Dmochowski *et al.* (*9*) observed some viruslike particles in electron micrographs of milk that lacked tumor-producing activity.

An earlier study from our laboratory (*10*) showed that viruslike particles are abundant in hyperplastic alveolar nodules of C3H/Crgl mice, as well as in their tumors, and occasionally appear in normal mammary tissues from the same strain. A recently developed technique has enabled us to test the neoplastic potentialities of normal and hyperplastic mammary tissues (*11*) and now has permitted us also to ask whether the viruslike particles demonstrable with the electron microscope appear regularly in tissues that can be classified as preneoplastic. The present report describes the consistent occurrence of these particles in hyperplastic mammary tissues—including some from so-called "virus-free" C3H sublines—that are neither normal nor neoplastic but preneoplastic. It also illustrates the apparent absence of similar particles in mammary hyperplasias artifically induced by hormone administration. The data suggest a consistent relationship between the occurrence of large numbers of viruslike particles and the development of mammary hyperplasias of preneoplastic significance.

MATERIALS AND METHODS

Hyperplastic alveolar nodules from 5 sublines of mice varying in their tumor incidence and outgrowths from transplanted nodules were examined in this study. Outgrowths from transplanted normal mammary tissues served as controls, and spontaneous mammary tumors were available for comparison. In addition, the normal hyperplasias of pregnancy (lobules of prelactating gland) and mammary "noduloids" induced by the administration of estrogen or cortisol (*12*) were examined to compare hyperplasias that are not preneoplastic with those of known preneoplastic status.

The transplantation technique, described in detail elsewhere (*11*), involves the removal of the mammary-gland anlage from one or both of

the #4 mammary fat pads of 3-week-old female mice and the implantation of chosen samples of tissue from donor mice into these gland-free fat pads. Within 8 to 12 weeks the transplanted tissues typically fill the fat pads with characteristic glandular structures and then may be compared with the host's own glands or with other mammary tissues transplanted contralaterally.

The following tissues were examined:

1. Hyperplastic alveolar nodules from old, nonpregnant, multiparous, tumor-bearing C3H/Crgl females: one or more nodules from each of 19 animals, including those discussed by Pitelka *et al.* (*10*).

2. Hyperplastic alveolar nodules from old, nonpregnant, multiparous C3H/Crgl/2 females: one or more nodules from each of 4 animals. This strain arose spontaneously from our C3H/Crgl stock and is distinguished from it by a relatively low incidence and late age of onset of mammary cancer (approximately 15% at 20 months).

3. Hyperplastic alveolar nodules and tumor from an old, nonpregnant, multiparous C3Hf/Crgl female. This animal was from the seventh generation of a "virus-free" line established by foster nursing of C3H/Crgl young on a BALB/cCrgl mother. The line has a very low tumor incidence; statistics are not yet available.

4. Hyperplastic alveolar nodules from an old, nonpregnant, multiparous female of a C3Hf subline obtained from the laboratory of Dr. W. E. Heston. This "virus-free" line, developed by foster nursing of cesarean-delivered C3H/He young on a C57BL mother has a tumor incidence of 30 to 50 percent at 20+ months.

5. A hyperplastic alveolar nodule from an old, nonpregnant, multiparous female of a C3Hf subline obtained from the laboratory of Dr. H. B. Andervont. This line developed after foster nursing of C3H/An young on a C57BL mother and has a tumor incidence of 20 to 40 percent at about 20 months.

6. Hyperactive outgrowths from nodules transplanted as described, where host and donor were both C3H/Crgl females: one or more samples of outgrowths from each of 22 transplants taken 8 to 12 weeks after initial implantation or after the third to eighth generation of serial transplantation.

7. Hyperactive outgrowth from a nodule from a C3Hf/He animal transplanted into a C3Hf/Crgl host.

8. Normal outgrowths from transplanted normal lobules, ducts, and end-buds: one or more samples of outgrowths from each of 25 transplants taken 8 to 26 weeks after implantation. In 18 cases host and donor were C3H/Crgl females, in 7 cases host and/or donor were C3Hf/Crgl or C3H/Crgl/2.

9. Normal lobules of prelactating gland from pregnant C3H/Crgl females: one or more samples from each of 28 animals, including those reported by Pitelka *et al.* (*10*) and Wellings *et al.* (*13*).

10. "Noduloids" induced by estrogen or cortisol. Four 10-week-old virgin C3H/Crgl females each received a 4 mg. pellet of estradiol-17β subcutaneously. After 30 to 45 days, the animals were injected with 100 μg. cortisol acetate, 100 μg. mammotropin, and 100 μg. somatotropin daily for 3 days for the purpose of inducing lactation in the "noduloids" but not in normal tissue (*14*). This permitted the accurate selection of "noduloids" for biopsy. True nodules, though not common in animals of this age, could begin to appear after the hormone treatment; any true nodules present would be indistinguishable from "noduloids" at biopsy, since they also lactate in response to the lactogenic treatment. One 10-week-old virgin C3H/Crgl female—other females in this group failed to produce detectable "noduloids"—was injected subcutaneously with 250 μg. cortisol acetate daily for 30 days, after which the lactogenic mixture was injected for 3 days before biopsy. Two or more "noduloids" were examined from each of the 5 animals.

Table 1 summarizes the tissue types and the results of electron-microscope examination for presence or absence of viruslike particles.

Table 1.—Summary of results from electron-microscope examination of hyperplastic tissues

Tissues examined	Strain of mice	Number of specimens	Viruslike particles
Hyperplastic alveolar nodules*	C3H/Crgl	19 animals, 1 or more nodules each	Abundant
Hyperplastic alveolar nodules	C3H/Crgl/2	4 animals, 1 or more nodules each	Abundant
Hyperplastic alveolar nodules	C3Hf/Crgl	2 nodules from 1 animal	Abundant
Hyperplastic alveolar nodules	C3Hf/He	2 nodules from 1 animal	Abundant
Hyperplastic alveolar nodules	C3Hf/An	1 nodule	Abundant
Hyperactive outgrowth*	Donor: C3H/Crgl Host: C3H/Crgl	22 outgrowths, 1 or more samples each	Abundant
Hyperactive outgrowth*	Donor: C3Hf/He Host: C3Hf/Crgl	1 outgrowth	Abundant
Normal outgrowth	Donor: C3H/Crgl, C3Hf/Crgl or C3H/Crgl/2 Host: C3H/Crgl or C3H/Crgl/2	25 outgrowths, 1 or more samples each	None or few (2 abundant)
Normal prelactating lobules	C3H/Crgl C3Hf/Crgl C3H/Crgl/2	28 animals, 1 or more samples each	None or few
"Noduloids"	C3H/Crgl	5 animals, 14 "noduloids"	None or few (2 abundant, probably true nodules)

*Similar tissues have been used in experiments to test tumor-producing potential and have been shown to be preneoplastic.

The design of this study required that large numbers of tissues be processed and examined in the electron microscope with maximum efficiency; the primary questions to be answered were: (*a*) Are viruslike particles present? (*b*) If so, where are they found in the cells? Answering these questions does not demand the highest resolving power of the microscope, hence moderately thick sections could be used, thereby increasing the probability of finding particles while still permitting accurate identification of most cell structures. Unlike mammary tumor and lactating normal gland, the immature, prelactating, and hyperplastic mammary tissues are extraordinarily unphotogenic. This is caused in part by the large intracellular and extracellular fat content, which may interfere with uniform thin sectioning, and in part by the typically dense, fibrogranular cytoplasm, which reduces optical contrast between the matrix and its inclusions. In spite of considerable experimental variations in technique, no procedure has been found that consistently yields tissues in an optimal state of preservation, though mammary tumor and lactating gland are very adequately prepared by some of the same methods.

The most satisfactory method, and one that appeared to achieve a compromise among the various technical problems, involved fixation of tissues from anesthetized animals in cold 1 percent osmium tetroxide buffered to *p*H 7.2 to 7.4, a brief washing, and then a leaching period of

2 to 7 days in 70 percent ethanol. This was followed by a 1-hour bath in 1 percent phosphotungstic acid in 70 percent ethanol, and then by rapid dehydration, impregnation with *n*-butyl methacrylate containing 2 percent Luperco CDB as a catalyst, and polymerization of the plastic at 60° C. Sections were cut on a Porter-Blum microtome, with plate-glass or diamond knives. Thick sections were stained with iron hematoxylin and examined by light microscopy. After identification of an area of glandular epithelium in the thick section, each block was trimmed to include such an area; thin sections were cut, mounted on Formvar film, and examined in an RCA EMU2C or an RCA EMU3E microscope.

RESULTS

An outgrowth from a normal lobule transplanted into a gland-free fat pad and allowed to grow for 10 weeks is shown in figure 1, which is a low-power photomicrograph of a stained wholemount preparation. The fat pad is nearly filled with normal gland that is in the same developmental stage as the virgin host's own intact glands. The outgrowth from a nodule transplanted at the same time into the contralateral #4 fat pad of the same host is shown in figure 2. It has a lobuloalveolar structure indistinguishable from that of a normal late-prelactating gland and is distinctly hyperactive relative to the normal host glands and to the normal outgrowth. Variations in the morphology of hyperactive outgrowths and their behavior when serially transplanted are discussed elsewhere (*15*). It has been shown that tumors arise much more frequently and in a much shorter time within nodule outgrowths than within normal outgrowths (*11*, *15*). The outgrowth as a whole, however, is not a tumor nor does it transform *per se* into a tumor; it constitutes a hyperplasia that may be preneoplastic but is not neoplastic.

When examined with the electron microscope, the outgrowth from a normal transplant in a virgin host shows the fine structural characteristics of normal virgin gland (*13*). Figures 3 and 4 show a section taken from the outgrowth of a normal duct 8 weeks after transplantation into a gland-free fat pad. This section passed through an area of active growth (mitotic figures were seen in neighboring regions), presumably of an end-bud or terminal duct. Cells are tall and slender, with varying numbers of irregular microvilli on their luminal surfaces. Nuclei show a rather uniformly dense peripheral layer and contain nucleoli; they occasionally are indented or lobed. The cytoplasm contains an abundance of finely filamentous and granular material, giving it a relatively dense appearance. There are numerous small, dense mitochondria, and the fine vesicular component of the Golgi zone is noticeable, often at more than one location in a single cell, as is characteristic for this tissue. No evidence of secretory activity is seen in the cytoplasm; the lumen appears empty.

Outgrowths from transplanted nodules have a fine structure comparable to that of a prelactating normal gland (*13*) in any stage from very early

pregnancy to term. Figure 5 shows parts of several cells and a lumen from a hyperactive outgrowth. General cytologic features are similar to those of the cells seen in figures 3 and 4, but secretory activity is indicated by the presence of several fat droplets in the cells and lumen and of protein particles in the lumen (*16*). In addition, several A particles are seen scattered in the apical cytoplasm and in microvilli. No complete B particles appear in this figure, but many were in the lumina in adjacent areas of the same tissue.

Figure 5 was selected to illustrate the normal, healthy appearance characteristic of the cells of most hyperactive outgrowths. Figures 6 to 10 give more detail of the appearance and distribution of viruslike particles. In the center of figure 6, well within the cytoplasm of one of the cells, are several large membrane-bound vesicles containing numerous encapsulated particles. A close look reveals that those in the center of the vesicle are typical B particles, with an outer membrane and 1 or 2 dense nucleoids. Near the periphery of the vesicle the particles frequently lack the nucleoid but show a dense inner ring within the outer limiting membrane [also called B particles by Bernhard (*1*)]. This inner ring is of about the same density and dimensions as those of the A particles seen lined up in a more or less orderly fashion on the cytoplasmic side of the vesicle membrane. Similar, smaller vesicles, usually enclosing encapsulated particles and always associated with clusters of A particles, occurred in several other places in the cytoplasm of these cells.

Figure 7 shows a group of intracytoplasmic vesicles surrounded by A particles in the upper cell, and a large cavity associated with many viruslike particles in the lower cell. This cavity may be part of a lumen, but more probably represents a fusion of a number of smaller intracellular vesicles. A rather long series of sections was cut through this region, all showing the cavity surrounded by the cytoplasm of a single cell; its membrane lacks the microvilli usually seen on luminal cell margins—furthermore, coalescence of a few intracytoplasmic vesicles is evident in some of our other micrographs.

In figures 8 and 9 are seen the luminal borders of 2 alveolar cells from a hyperactive outgrowth that contained abundant, macroscopically visible, milky secretion, though the virgin host possessed undeveloped mammary glands. As in all other instances of apparently "lactating" outgrowths, secreted material was seen in the cells and lumina of this tissue, but cell ultrastructure was that of late prelactating, rather than true lactating, gland. Particularly characteristic of these "pseudolactating" outgrowths, where large masses of intracytoplasmic A particles have not been found, is the appearance of what may represent incomplete A particles at the luminal border of the cell. These formations are much like the structures demonstrated by Lasfargues *et al.* (*3*) and Moore *et al.* (*4*) in young cultures of RIII mammary-tumor cells *in vitro*.

Figure 10 is a low-magnification micrograph of an alveolus from a typical hyperactive outgrowth. There are B particles in the lumen and small to very large masses of A particles, some enclosing vesicles, in every cell sur-

rounding it. Such an abundance of viruslike particles is not unusual in hyperactive outgrowths.

In an earlier report (*10*) we showed that viruslike particles were abundant in hyperplastic alveolar nodules. Many additional nodules have been examined since the publication of this report; in every one, viruslike particles were readily demonstrated. Figure 11 is selected to show clustered intracytoplasmic A particles, A particles in microvilli, and free encapsulated B particles without nucleoids, all within a small area of a nodule. Like the outgrowths from transplanted nodules, the nodules themselves appear to be composed usually of healthy, active cells; varying amounts of secreted material are present.

We have studied 4 low-tumor C3H sublines (C3Hf/He, C3Hf/An, C3Hf/Crgl, and C3H/Crgl/2) which, on the basis of standard biologic tests, are assumed to be free of the mammary-tumor virus. Nonetheless, old parous females from these lines bear hyperplastic alveolar nodules and these nodules, as shown in a representative micrograph (fig. 13), contain numerous viruslike particles. Figure 12 shows a section through the hyperactive outgrowth from one such nodule from the "virus-free" C3Hf/He subline transplanted into a gland-free C3Hf/Crgl fat pad. Again, viruslike particles are present. In the morphology, distribution and abundance of viruslike particles, as well as in cell ultrastructure, hyperplastic tissues from the low-tumor C3H sublines do not differ discernibly from those of C3H/Crgl mice.

If viruslike particles are in some manner a *result* of hyperplastic growth, or if a region of rapidly growing alveolar cells is simply a favorable site for the invasion and multiplication of viruslike particles, then an abundance of such particles is to be expected in other mammary hyperplasias. To test this we have examined (*a*) the normally occurring hyperplasia that constitutes the prelactating lobules of pregnancy, and (*b*) "noduloids" induced in the mammary glands of young, nonpregnant females by the administration of estrogen or cortisol (*12*).

As previously noted (*10*, *13*) viruslike particles are found occasionally in normal prelactating mammary tissue. They occur as scattered B particles in the lumen or as rare clusters of A particles in the cytoplasm. We have found them in normal tissues of perhaps 25 percent of the pregnant animals examined (including those from low-tumor sublines), but only in an occasional section of these tissues and never in large numbers. It seems probable that viruslike particles are fairly common, though not in high concentration, in normal prelactating gland, but that the inadequate sampling methods of electron microscopy detect them in only a relatively small percentage of individuals. By contrast, particles are readily seen in nearly every section surveyed of nodules, hyperactive outgrowths, and tumors, provided that an appropriate area of apical cytoplasm or of lumen is included.

Among the 25 outgrowths from normal transplants sampled for this study, 18 were biopsied while the hosts were in the latter half of pregnancy. These animals were 5 to 7 months old and therefore within the age range

when nodules containing high concentrations of particles can occur. The outgrowths showed a fine structure indistinguishable from that of normal prelactating glands, and few or no viruslike particles were found in 16 of them. In 2 individual outgrowths, however, there were numerous viruslike particles in several tissue samples taken from different parts of the gland. Because hyperactive outgrowths are macroscopically similar to normal prelactating gland it was not possible, by examining either the living animal at the time of biopsy or the wholemount prepared from the remaining outgrowth, to characterize these outgrowths as anything different from normal prelactating gland. In 1 of these 2 cases, outgrowth from a normal transplant in the contralateral #4 fat pad of the same host was available and showed no viruslike particles. In the same case, control tissue samples had been taken by biopsy 16 weeks earlier, when the outgrowth was only 8 weeks old, and these showed all the characteristics of normal virgin gland, without any detectable viruslike particles.

These 2 examples, which suggest a development of viruslike particles after the establishment of an outgrowth, are unprecedented in our experience. Experiments designed to explore the questions they pose are being initiated. For the present, we emphasize that this has occurred in only 2 out of a total of 25 transplants of normal tissue, and our conclusion that the normal hyperplasias of pregnancy are not characterized by an abundance of viruslike particles is unchanged.

"Noduloids" induced by 30- to 45-day treatment with estradiol-17β or with cortisol acetate consist of isolated groups of alveoli, which resemble lobules of prelactating mammary gland (*12*). They differ from true nodules in that they often appear abundantly in young animals after this high but relatively short-term dosage with the hormone, and unlike nodules they usually regress after treatment stops. As far as is known, "noduloids" *per se* have no preneoplastic significance; when transplanted into cleared fat pads, they give rise to outgrowths that are not hyperactive.

After the brief lactogenic treatment in the present study, the estrogen-induced "noduloids" were inflated with whitish secretion and grossly resembled early lactating lobules. In electron micrographs, epithelial cells were flattened, and fat and protein particles appeared in the cytoplasm and filled the lumen (figs. 14, 15, and 16). Of the 12 different presumed "noduloids" from estrogen-treated animals, 10 conformed to this description and contained no viruslike particles or only a few scattered ones, as in normal prelactating tissue. The remaining 2, from the same female, contained large numbers of particles. They were composed of large cells, with rather diffuse cytoplasm and no organized ergastoplasm. Two other "noduloids" from the same female were typical of the majority of the estrogen-induced hyperplasias. We feel justified in assuming that the 2 particle-containing tissues were true nodules. The host was 4 months old at the time of biopsy and had received estrogen for 45 days. Since estrogen treatment also increases the incidence of true nodules (*12*), the selection of an occasional nodule under these conditions is not surprising.

A point of some interest in connection with ultrastructure of secreting

cells in general is the presence of conspicuous arrays of organized ergastoplasmic membranes in some cells of all samples examined of the 10 typical, lactating, estrogen-induced "noduloids." Although somewhat less abundant than in lactating normal mammary gland (*13*, *17*), these membranes are much more common than in prelactating normal tissue, or in any of the hyperplastic nodules and outgrowths. Among these nodules and outgrowths are some that grossly appear to be lactating, since they are distended with white fluid, even in a virgin host. This apparently is a permanent condition; it has been maintained in hyperactive outgrowths through several serial transplant generations. Electron micrographs of these tissues have consistently revealed an ultrastructure characteristic of a late prelactating gland; cells are low-cuboidal and ergastoplasmic membranes are sparse, with parallel alignment occurring only in occasional limited areas (fig. 17). It may be significant that in these permanently "lactating" hyperplasias the situation is perforce a static one; milk once secreted into the lumina can only be removed by resorption since suckling is not taking place and in the case of outgrowths, which lack a nipple connection, could not take place. Thus, secretion in significant quantities is probably not continuous. The "noduloids," on the other hand, were biopsied after only 3 days' exposure to a lactogenic hormone mixture. We do not know how long the ultrastructure characteristic of lactation would have been maintained if lactogenic treatment had been continued. It is also possible that the prominent ergastoplasm is a consequence of the estrogen treatment itself, since the estrogen pellet is still exerting its influence during the lactogenic treatment.

The lactogenic treatment was largely ineffective in the cortisol-treated mouse. Here the "noduloids" (fig. 18) were cytologically similar to normal gland in midpregnancy. Alveoli had relatively small lumina surrounded by large cuboidal cells. There was much secreted material in some "noduloids" and less in others. Occasional cells contained limited zones of oriented ergastoplasmic membranes. No viruslike particles were seen.

DISCUSSION

Two well-known facts concerning mouse mammary cancer should be recalled: (1) A high incidence of spontaneous mammary tumors occurs only if the mammary-tumor virus is present, and (2) this virus can persist and multiply in the absence of detectable tumors, since a lactating female may transmit it in her milk before developing a cancer. Both the sporadic occurrence of viruslike particles in normal mammary tissues and their abundance in at least some mammary cancers are observations that are not surprising, if the viruslike particles are identical with or diagnostic of infection with the mammary-tumor virus.

However, the presence of abundant viruslike particles in the presumed "virus-free" C3Hf sublines is surprising and requires further consideration. Cesarean delivery of a single litter of C3H/He young, followed

by foster nursing of this generation only on a C57BL/He female, gave rise to the C3Hf/He subline (*18*) and the same procedure has been used repeatedly to obtain other "virus-free" individuals or sublines. These sublines typically show an abrupt and permanent drop in mammary-tumor incidence and rise in mean age of onset as compared with the parent lines [*see* (*19*) for statistics]. Furthermore, no tumor-producing activity is demonstrable in their milk or in cell-free filtrates of their mammary tumors (*19*). Upon reinfection of a C3Hf individual with mammary-tumor virus, high incidence and early onset of mammary tumors are re-established in her descendants. The C3H/Crgl/2 subline arose spontaneously from a single C3H/Crgl female and, like the C3Hf sublines, has a reduced tumor incidence at an advanced age. In all of these instances old parous females bear hyperplastic alveolar nodules, and in all of these nodules that we have examined there are viruslike particles in abundance, indistinguishable from those of the C3H/Crgl strain.

Ultimately a choice will have to be made among the alternative possibilities that (1) the viruslike particles are a morphologically apparent phase of the mammary-tumor virus; (2) the viruslike particles are non-viral products of the metabolism of hyperplastic and some neoplastic cells; or (3) the viruslike particles are a virus but not the conventional mammary-tumor agent. If the first possibility is true, then the conflicting data presented by the C3Hf lines will have to be explained. It does not seem reasonable at present to account for their *sustained behavior over many generations* on the basis merely of an initial sharp drop in concentration of the tumor virus or by a sharp increase in a proposed inhibitor (*4*). The second possibility is weakened by the fact that particles do not appear in significant numbers in normal or hormone-induced hyperplasias. This has been true in the overwhelming majority (63 out of 67) of such tissues we have examined. We should like now to consider to what extent the existing evidence favors the third possibility.

That there is no directly proportional relationship between speed of mammary-tumor growth and number of viruslike particles detectable in it by electron microscopy was demonstrated by Bernhard and Guérin (*20*). They found comparable variability in particle appearance in spontaneous tumors from high- and low-cancer strains of mice, and in some transplanted tumors. They also reported that one tumor, after a history of serial transplantation and tissue culture, was highly malignant but could not be shown to contain any particles at all.

Nodules and their outgrowths—which in our experience *always* contain many particles—represent cell populations altered from normal in such a way that they are more responsive to endogenous hormones than unmodified cells and therefore capable of hyperplastic growth in a normal environment. These hyperplasias are not themselves tumors, but some of them will give rise to tumors in the ordinary course of events. In the tumors so evolved, viruslike particles may be as abundant as in nodules or their hyperactive outgrowths, but not necessarily more abundant.

It appears that prolific particle multiplication occurs early in the course of transformation from the normal state and that it coincides with the formation of nontumorous hyperplasias (precancerous nodules). Particle production continues in the established hyperplasias and in at least some of the tumors that may arise within them, but to date we have no direct evidence that the particles are related to tumor production itself.

Indulging in an attempt to reconstruct a partial life cycle of the viruslike particles on the basis of strictly morphologic evidence, we run into some paradoxes, already recognized by Lasfargues *et al.* (*3*). These authors, as noted, consider that virus formation occurs solely at the free border of infected cells; since they found few concentrations of A particles in their RIII tumors and tumor cultures, they rejected these as possible stages in virus growth. The relationship of A to B particles in our material is apparent in the micrographs discussed and need not be reiterated here. Our data allow us to postulate the following scheme:

The particles multiply in a cell where little or no secretory activity is occurring; they accumulate in the cytoplasm in the form of A particles. These require a membrane contributed by the host cells for maturation, but in the absence of active secretion processes, the A particles are not carried to the cell surface; hence they induce the formation of intracytoplasmic membranes (perhaps they take advantage of Golgi membranes, though this is not demonstrated in our material). Mature particles then collect within vesicles bounded by these membranes. Those particles that happen to be near the apical cell border acquire their membranes from microvilli and are released into the lumen to complete their maturation into B particles. When secretion of milk or premilk commences, particles and their precursors are moved, like milk fractions, to the luminal border of the cell and released there rapidly enough so that large accumulations of A particles are rare. If "secretion" of particles is sufficiently rapid, young developmental stages at the cell border may be the only intracellular ones visible.

Whereas most of the data assembled until now have tended to support or at least to permit the logical and satisfying assumption that in electron micrographs we were seeing the mammary-tumor virus, the present report opens this assumption to serious question. Further study, for example of the rare nodules occurring in the breasts of mice of strains such as C57BL and BALB/c that are reputably free of the mammary-tumor virus, is called for. For the present, the clear and consistent association of viruslike particles with preneoplastic hyperplasias in C3H mice is established.

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PLATES

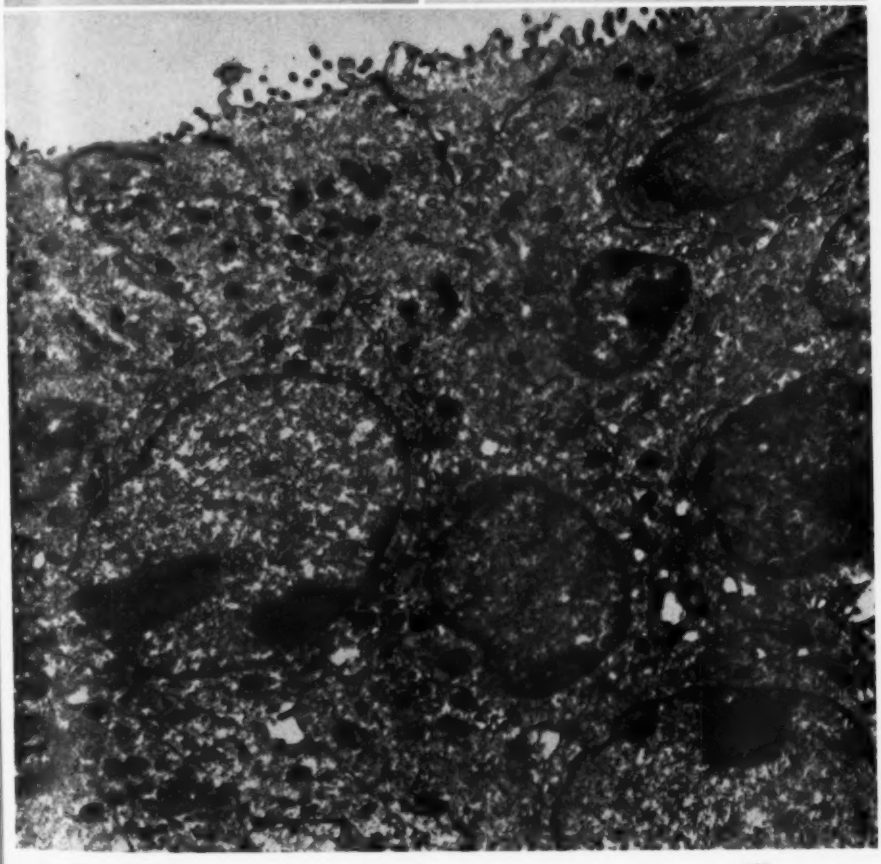
The line marker on all electron micrographs indicates 1 μ.

PLATE 106

FIGURE 1.—Photomicrograph of whole outgrowth from sample of normal mammary gland 10 weeks after transplantation into gland-free *left* #4 mammary fat pad of 3-week-old female. Outgrowth resembles normal virgin gland. L=lymph node. Hematoxylin. × 5

FIGURE 2.—Photomicrograph of whole hyperactive outgrowth from nodule 10 weeks after transplantation into gland-free *right* #4 fat pad of same 3-week-old female as figure 1. Outgrowth resembles a late prelactating gland. A tumor (T) has arisen at *upper* end of gland. Hematoxylin. × 5

FIGURE 3.—Low-power electron micrograph of section through end-bud or terminal duct from outgrowth of normal duct 8 weeks after transplantation. Small part of empty lumen, bordered by microvilli, appears at *top*. *Note* the columnar shape of cells, particularly evident in 2 occupying *left* half of picture. × 11,500

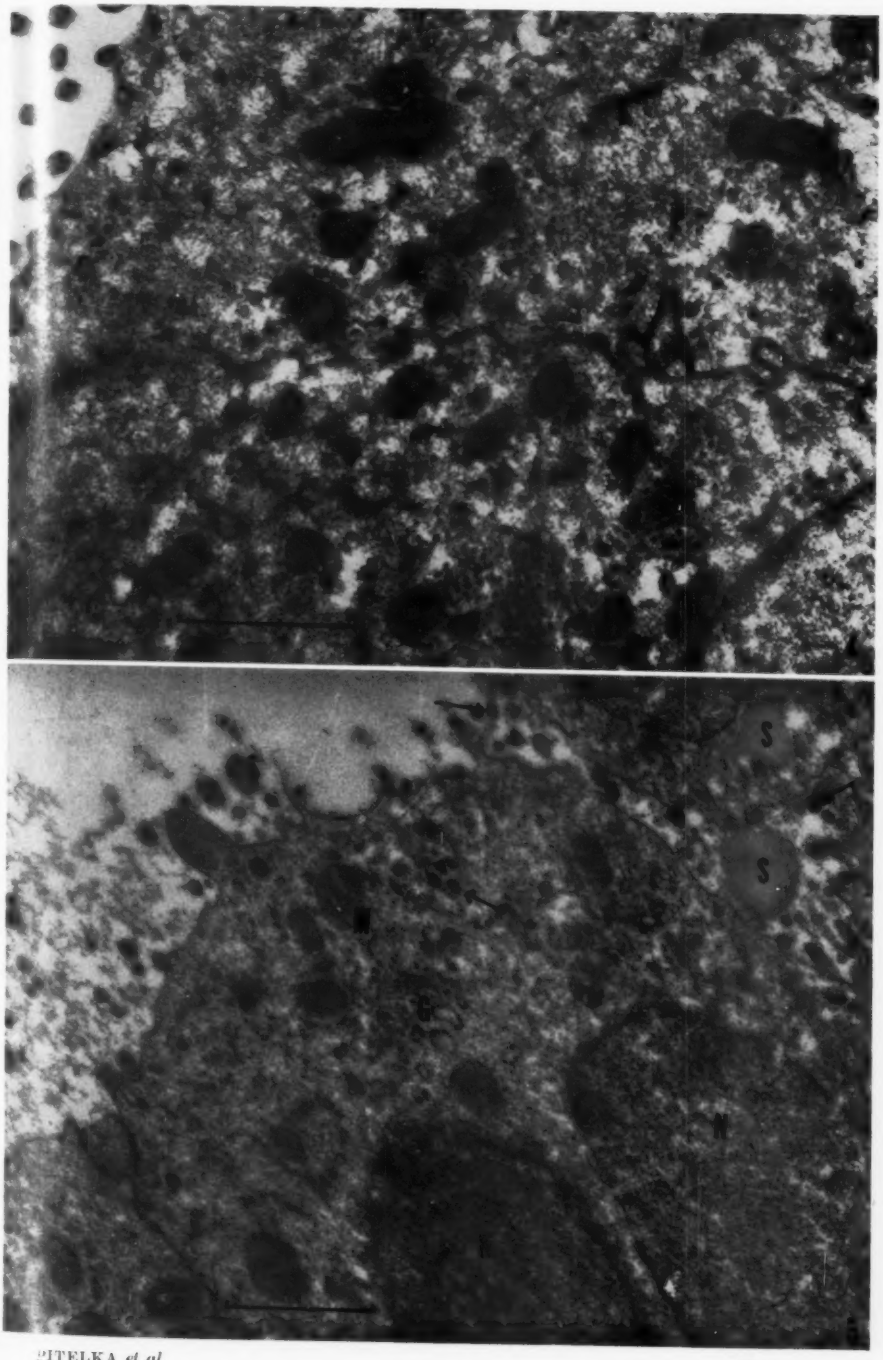


560070—60——9

PLATE 107

FIGURE 4.—Higher magnification of part of area seen in figure 3. Mitochondria (M) have dense matrix in which cristae are discernible. Golgi apparatus (G) appears as parallel rows of microvesicles. Cytoplasmic matrix is filamentous and finely granular, with occasional smooth vesicular or tubular profiles. Rough-surfaced double membranes (E) are rare. × 27,000

FIGURE 5.—Section through part of hyperactive outgrowth from nodule transplant. Cytoplasm and lumen contain droplets of secreted material (S). Mitochondria (M) have dense matrix; clusters of small vesicles probably represent tangential section of Golgi zone (G). Two lobes of the nucleus (N) are sectioned at *lower* right. Viruslike particles (*arrows*) are distinguished from other vesicular profiles in cytoplasm by uniform larger size and heavier contours. × 22,000



560070—60——10

PLATE 108

FIGURE 6.—Parts of several cells from hyperactive outgrowth from nodule transplant. At extreme *lower right* corner is edge of lumen (L) that is densely packed with viruslike particles and amorphous material. At *right center* are series of intracytoplasmic, membrane-bound vesicles containing B particles. Those in center of larger vesicles contain dense nucleoids; those nearer the periphery resemble encapsulated A particles. On the cytoplasmic side of vesicle membrane A particles are aligned in a nearly complete single layer. × 25,000

FIGURE 7.—Parts of several cells from hyperactive outgrowth from nodule transplant. In *upper right* is a group of intracytoplasmic, membrane-bound vesicles with A particles clustered around them. At the *lower left* is a larger cavity, perhaps resulting from fusion of a number of smaller vesicles. Because of angle of section, the membrane surrounding the cavity is indistinct here. B particles and encapsulated A particles appear in the cavity, and A particles are grouped in cytoplasm around it. × 22,000

FIGURE 8.—Luminal border of cell from hyperactive outgrowth that appeared grossly to be lactating in virgin host. Five evaginations of cell surface toward lumen are seen, with crescent-to-horseshoe-shaped thickenings immediately *below* cell membrane. Those at *right* suggest nearly complete A particles. × 63,000

FIGURE 9.—A field similar to figure 8, but from different "lactating" outgrowth. Thickenings appear at ends of microvilli. × 63,000

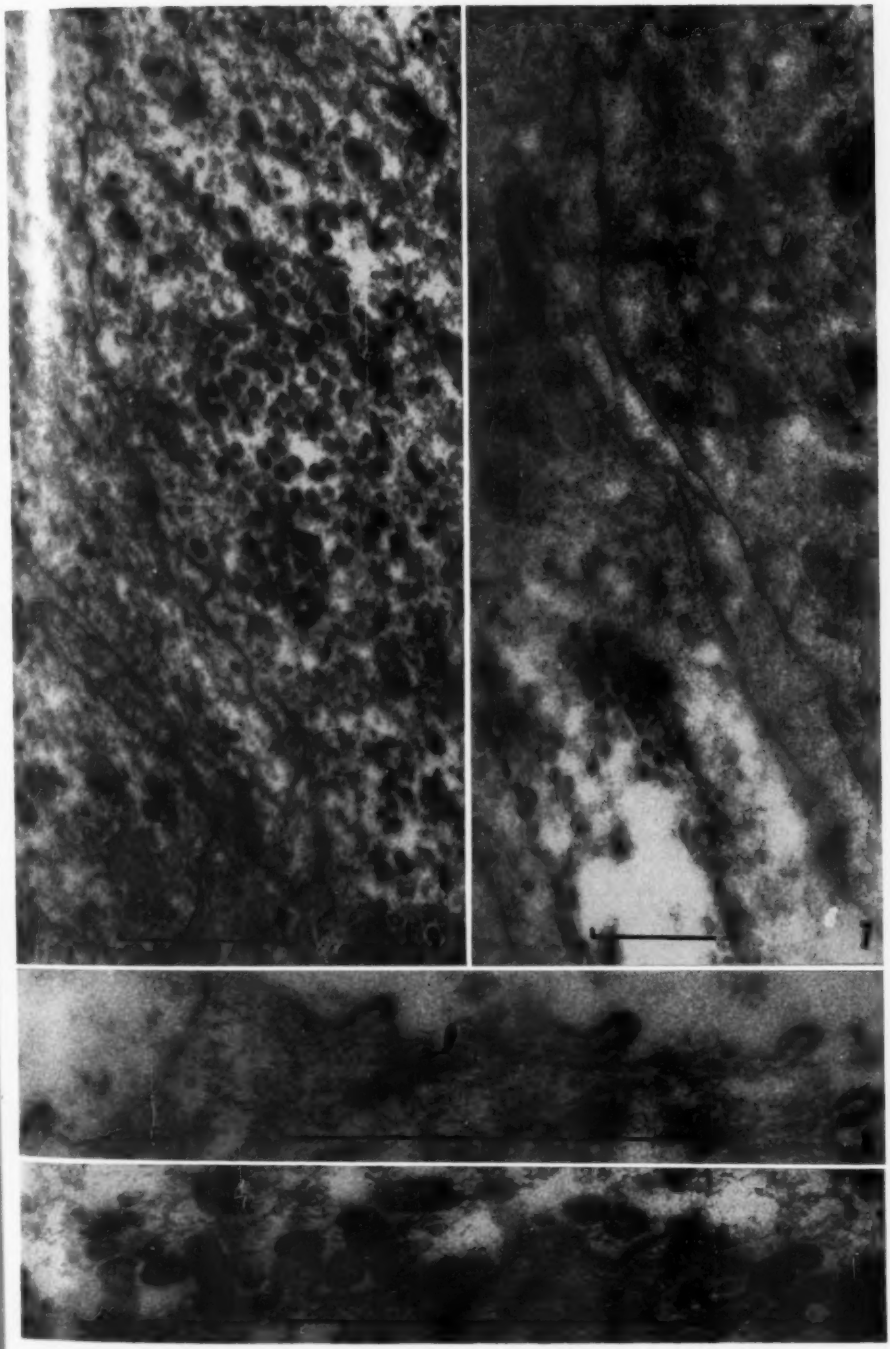


PLATE 109

FIGURE 10.—Low-power electron micrograph of small lumen and apical ends of surrounding cells from hyperactive outgrowth from nodule transplant. In this rather thick section viruslike particles appear very dark. In every cell surrounding the lumen, small to large clusters of viruslike particles are seen. × 12,000

FIGURE 11.—Small area of hyperplastic alveolar nodule from old C3H/Crgl female. The section cuts into a lumen in 2 places; at both borders are microvilli enclosing A particles, and some appear free in lumen. Clusters of A particles occur in cytoplasm. × 30,000

FIGURE 12.—Small area of hyperactive outgrowth from nodule transplanted from old C3Hf/He animal into gland-free fat pad of young C3Hf/Crgl host. Viruslike particles are present in cytoplasm, in microvilli at cell border, and in lumen. × 24,000

FIGURE 13.—Parts of 2 cells and lumen from hyperplastic alveolar nodule from old C3Hf/An animal. Edge of large, fat droplet appears at *lower left*. Clusters of A particles are visible at several locations in cytoplasm and in microvilli at luminal surface. × 24,000

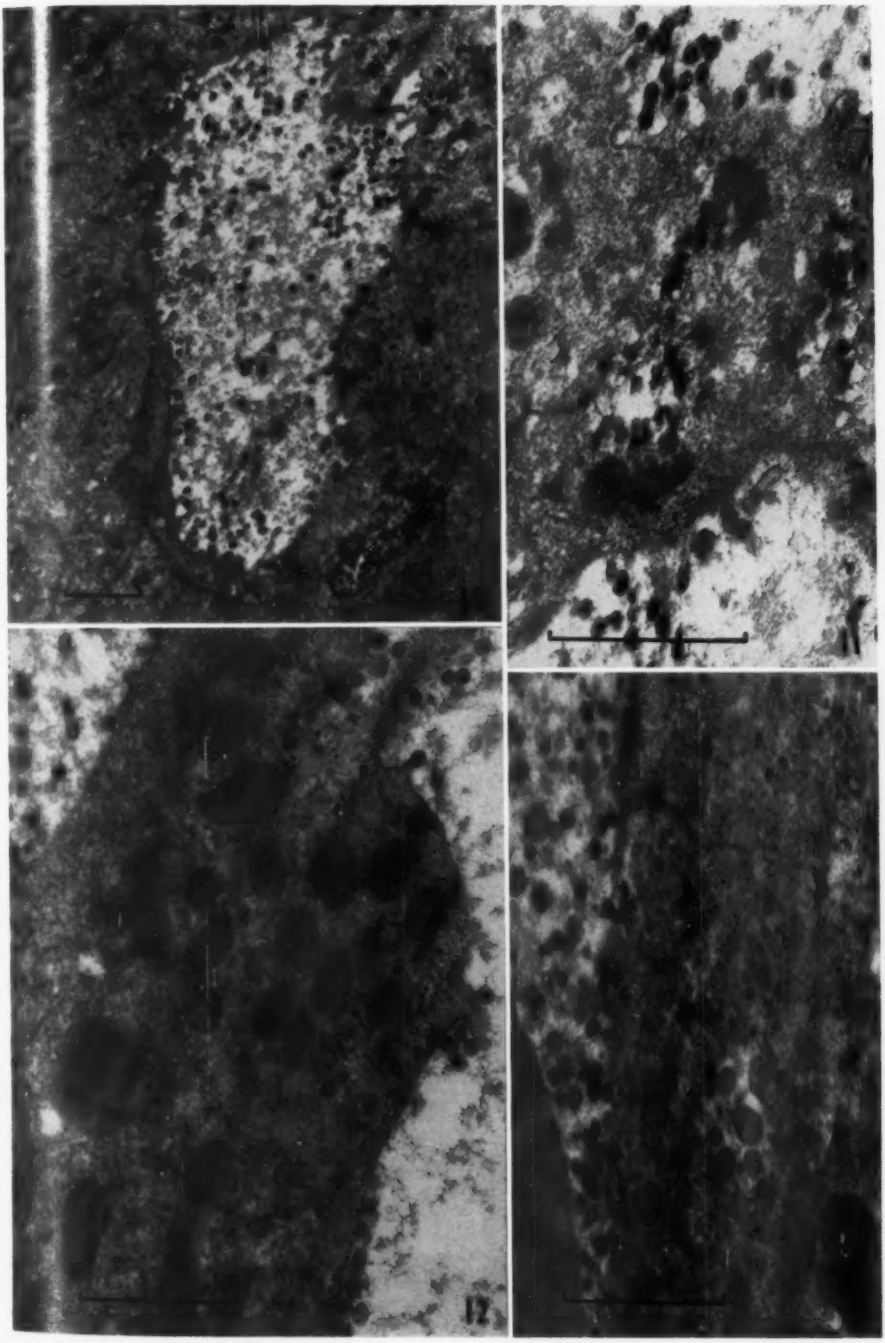


PLATE 110

FIGURE 14.—Low-power micrograph of part of an alveolus from an estrogen-induced "noduloid." Parallel arrays of ergastoplasmic membranes (E) occupy the basal regions of cells to the *left* and occur sporadically elsewhere. Cells are relatively flattened and large lumen is filled with secreted material. A large lipide droplet (S) appears in the cytoplasm. × 11,000

FIGURES 15 AND 16.—Small areas of apical cytoplasm of 2 cells from an estrogen-induced "noduloid," not from same animal as figure 14. *Note* secreted material (S) in cytoplasm and lumen, ergastoplasmic membranes (E) occurring even in apical region, and Golgi apparatus (G). Figure 15: × 24,000, figure 16: × 32,000.

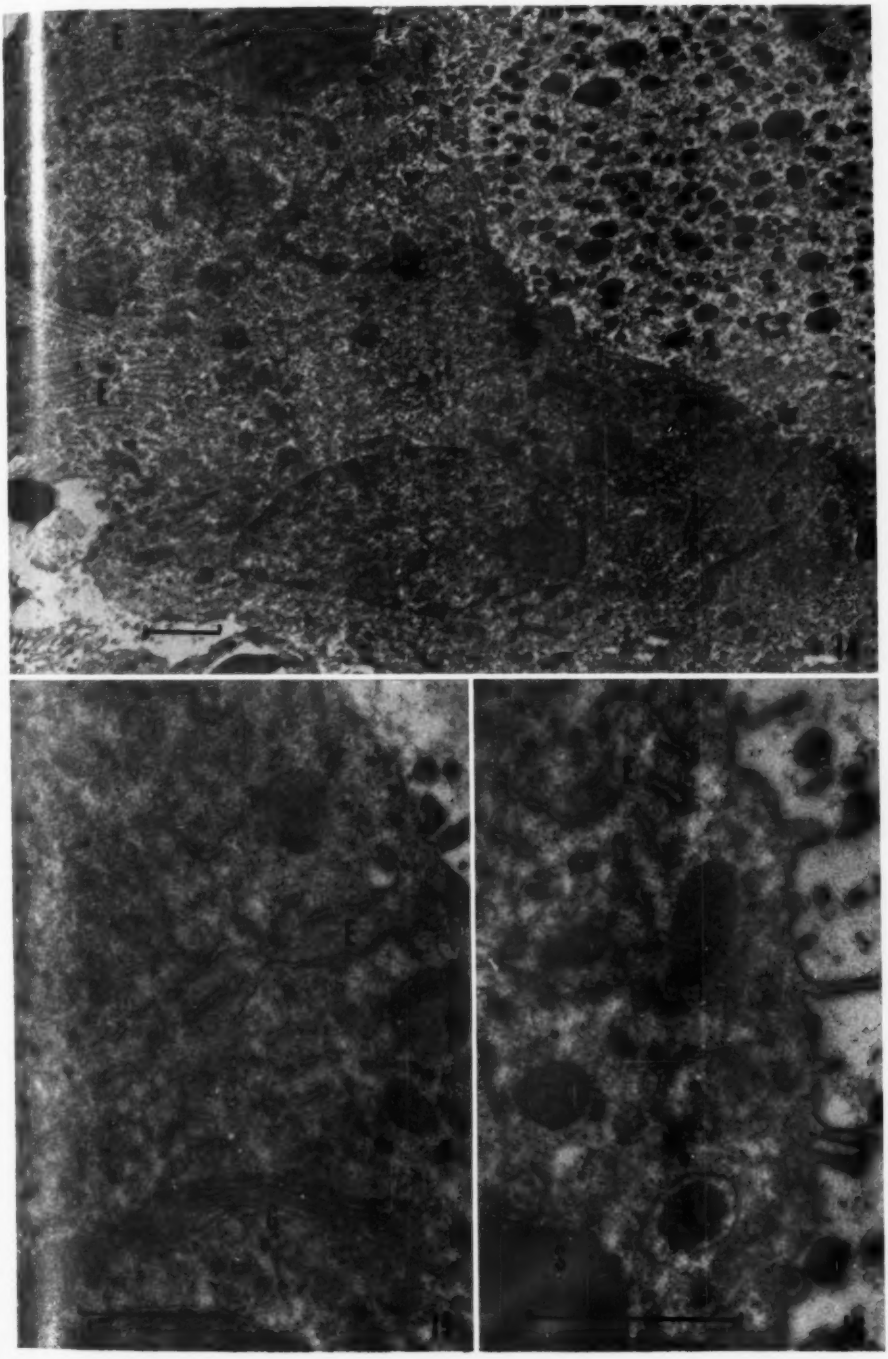
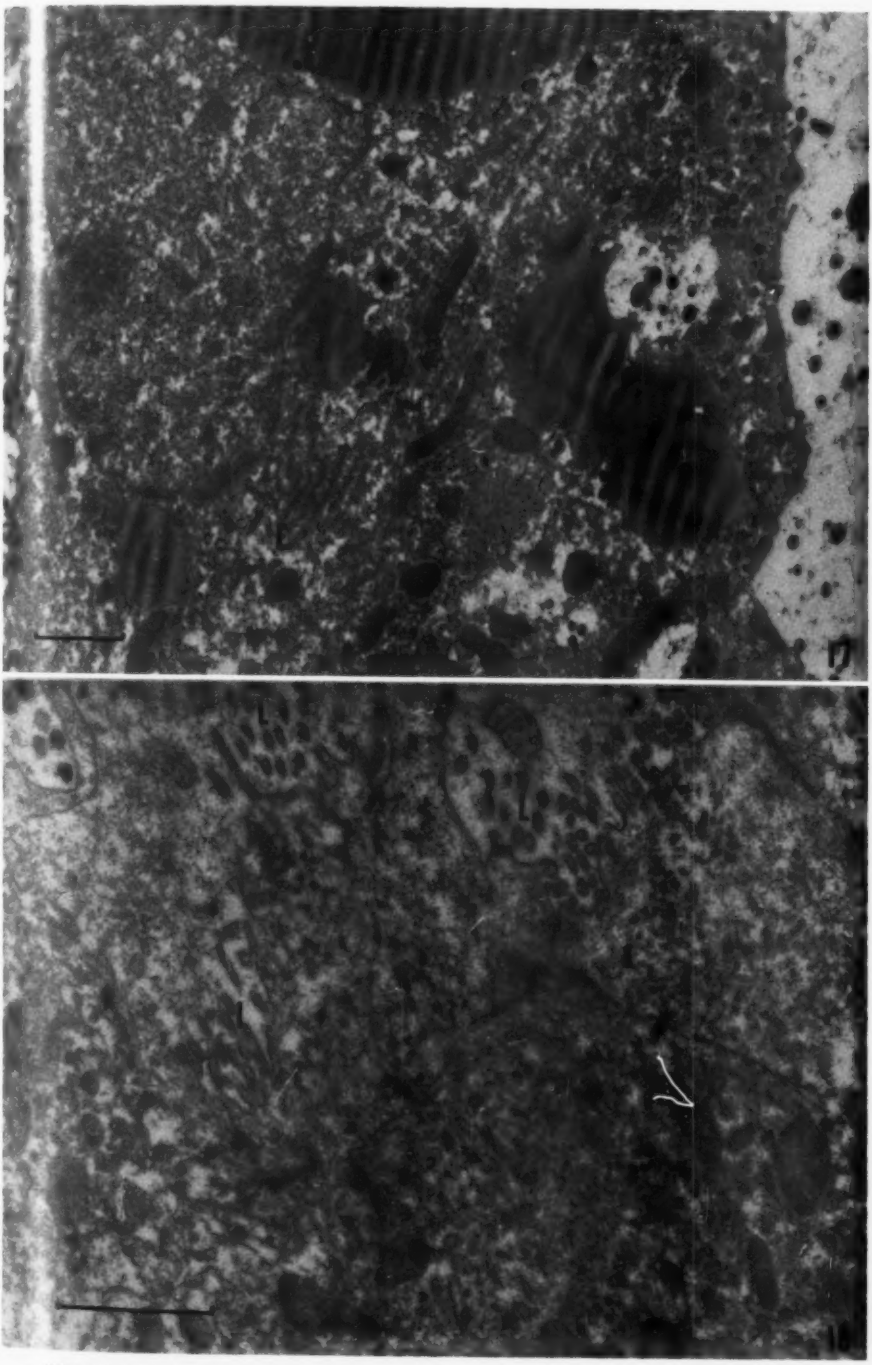


PLATE 111

FIGURE 17.—Small area from hyperactive outgrowth in 8th generation of serial transplantation after initial transplantation of nodule. This line has consistently maintained a "lactating" appearance in virgin hosts. Secreted material is abundant, particularly in the cytoplasm in sample. The zone of parallel ergastoplasmic membranes (E) near nucleus is as extensive as any ergastoplasm seen in these "lactating" outgrowths, but much less so than in estrogen-induced "noduloids" or in normal lactating gland. Viruslike particles are not apparent at this magnification but were numerous in higher-power micrographs of neighboring areas of same tissue. × 14,000

FIGURE 18.—Parts of several cells from cortisol-induced "noduloid." The cytoplasm contains many vesicular profiles similar to Golgi microvesicles but not to be confused with viruslike particles (*cf.* figs. 6 and 7). Cells are not flattened, and lumen (L) is irregularly constricted and in this sample contains little secreted material. Ergastoplasmic membranes are rare. × 24,000



Immunologic Response to Lymphocytic Choriomeningitis Virus in Lethally Irradiated Mice Treated With Bone Marrow [1]

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SUMMARY

Lymphocytic choriomeningitis-immune, nonimmune, and lymphocytic choriomeningitis-carrier mice were exposed to lethal total-body X irradiation and protected by an inoculation of marrow from either immune or nonimmune donors. Subsequent challenge of the surviving chimeras with potent homologous virus established that, under the conditions of these experiments, immunity was a function of the host and not of the transplanted tissue. These observations and those of other workers may be explained by assuming that the preirradiation immunologic response of the chimeras usually persists after irradiation, but that immunologically competent marrow may either react against the host or impose its own immunologic mechanism upon the host.—J. Nat. Cancer Inst. 25: 779–786, 1960.

THE PUBLISHED studies on the immunologic mechanism in *lethally* irradiated mice treated with bone marrow have dealt with nonliving antigens and readily demonstrable humoral antibodies (*1–3*). Several observations appear to support the view that the production of the humoral antibodies in these systems is a function of the irradiated host and not of the transplanted marrow (*1, 3*). Transplanted spleen cells, but not marrow, have been demonstrated to be immunologically competent (*2, 4*). From the evidence summarized by Hollingsworth (*3*), the indication is that marrow transplantation may transfer both hematopoietic and immunologically active tissue. Furthermore, genetic studies suggest that in "secondary disease" the grafted marrow reacts against specific histocompatibility antigens in the irradiated mouse (*5*). In a rat-rabbit chimera, La Via *et al.* demonstrated that the graft can actively engage in antibody formation (*6*).

The present investigations deal with irradiated mice, either immune [4] to lymphocytic choriomeningitis (LCM) virus or nonimmune, which were

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[4] Immunity as used in these experiments means ability of the mouse to withstand an otherwise fatal challenge with homologous virus.

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given marrow transplants from immune or nonimmune donors. After a suitable interval, these chimeras were challenged with fully potent homologous virus. Under the conditions of the experiments, the results indicate that immunity—which in mice infected with LCM does not depend upon circulating antibodies (*7*)—was determined by the host and not by the transplanted marrow.

With the evidence that noncirculating antibodies as well as circulating antibodies are a function of the host in these irradiation-induced chimeras, an attempt is made to incorporate the existing evidence, that the immune response may be of either host or donor origin, into a single working hypothesis.

MATERIALS AND METHODS

Animals.—Mice were from three sources. A colony of Swiss mice having acquired the viral infection before birth (*8*) were healthy, lifetime carriers of LCM. They were derived 11 years ago (30 generations) from the National Institutes of Health general purpose stock (noninbred Swiss mice) and have been maintained by cousin matings. These mice are designated "WCF," an appellation of convenience with no genetic significance. The (BALB/c × DBA/2)F_1 hybrids and noninbred Swiss mice were obtained from the National Institutes of Health animal-production unit.

Virus.—The LCM virus, other than that in the "WCF" colony, was from an intracerebrally passaged strain (*8*). For immunization, mice were inoculated subcutaneously with saline suspensions of infected mouse brain diluted to give 800 times the intracerebral LD50 for each subcutaneous inoculum. This produced an inapparent infection. Two weeks later the mice were challenged intracerebrally with 100 LD50 inoculum of infected brain suspension. The intracerebral challenge proved their immunity and simultaneously produced a hyperimmunity (*8*). When appropriate, viremia was determined by the intracerebral inoculation of heart blood (10^{-1}) into susceptible Swiss mice.

Irradiation.—Mice were 3 to 4 months old when exposed to a lethal X-ray dose of 800 r. Two X-ray tubes placed opposite each other were operated at 200 kvp and 15 ma.; filtration was 0.25 mm. Cu and 0.55 mm. Al with 54 cm. from each focal spot to the center of the mouse. The dose rate was 120 r per minute. Mice were irradiated in groups of 5 to 10 animals in a lucite box and were not randomized after irradiation.

Marrow inoculation.—Bone marrow suspension was prepared as previously described (*9*). Marrow from both femora and both humeri of each donor was suspended in 0.5 ml. of Locke's solution and used for one recipient. All inoculations into the tail vein were completed within 3 hours after irradiation. Throughout the experiments, mice were kept in glass battery jars and fed enriched whole-wheat bread soaked in reconstituted dried whole milk.

RESULTS

As a preliminary step, bone marrow transplantation was studied in lethally irradiated mice of the "WCF" colony which are congenital carriers of the LCM virus. The object was to determine whether marrow from hyperimmunized donors would terminate the infection in irradiated mice from this colony.

Since all mice of the "WCF" colony harbor virus, the marrow donors were Swiss mice from the general purpose stock of the National Institutes of Health. The hosts and donors were separated by 30 generations of cousin matings. Only 3 mice of the 42 irradiated animals inoculated with bone marrow survived more than 20 days (total of 2 experiments). None of the irradiated noninoculated control mice survived beyond the 15th day. Hyperimmunizing donors with crude tissue preparations containing the LCM virus may have immunized the marrow donors to tissue antigens in the irradiated host and may have caused the early death of the treated mice (*10*).

During the experiments, viremia tests on the survivors were all positive: 8 on the 12th day, 4 on the 19th, 2 on the 42d, and 1 on the 58th. One mouse dying on the 41st postirradiation day had LCM virus, in a pool of liver, spleen, and kidney tissue. All these mice had received marrow from hyperimmune donors. Therefore, under the conditions of the experiment, there was no alteration in the existing viremia.

In subsequent experiments (BALB/c × DBA/2)F_1 hybrids were used as irradiated hosts and marrow donors. An intraperitoneal inoculation of spleen cells was given in addition to the intravenous inoculation of bone marrow after irradiation. In the first experiments with the F_1 hybrid mice, the isologous transplants, all from hyperimmune donors, protected the recipients from irradiation death but did not confer on them the ability to withstand the intracerebral challenge with LCM 22 days later (table 1).

The second experiment was designed to test whether marrow from nonimmune donors would invoke loss of previously induced immunity in irradiated mice. The results are presented in table 2. Bone marrow from nonimmune donors protected 9 of 12 immune mice from irradiation death. Seven of these 9 survivors withstood LCM challenge (expt. group E). Although the dose of virus used for the challenge was potent

TABLE 1.—(BALB/c × DBA/2)F_1 hybrid mice protected after lethal irradiation by inoculations of marrow and spleen from hyperimmune hybrid donors

Expt. group	Number of mice	X ray	Hyperimmune marrow and spleen*	Challenged† Died/Injected
A	15	—	None	15/15
B	5	—	Intravenous, intraperitoneal	5/5
C	15	800 r	Intravenous, intraperitoneal	15/15
D	9	800 r	None	0/0‡

*Marrow given intravenously, spleen intraperitoneally.
†Challenged on 22d day.
‡ Died of acute irradiation damage.

TABLE 2.—Hyperimmune and nonimmune (BALB/c × DBA/2)F_1 hybrid mice protected after lethal irradiation by inoculation of marrow and spleen from nonimmune or hyperimmune hybrid donors

Expt. group	Number of mice	X ray	Immune status of		Challenged*
			Host	Donor	Dead/Injected
A	5	—	Nonimmune	—	5/5
B	5	—	Hyperimmune	—	0/5
C	12	800 r	Nonimmune	Nonimmune	2/6
D	12	800 r	Nonimmune	Hyperimmune	1/1
E	12	800 r	Hyperimmune	Nonimmune	2/9
F	5	800 r	Hyperimmune	—	0/0†

*Challenged on 21st day.
†Died of acute irradiation damage.

enough to kill all 5 nonirradiated, nontransplanted, nonimmune control mice (expt. group A), 4 of 6 nonimmune mice protected with nonimmune marrow survived the challenge with LCM (expt. group C). This survival of irradiated nonimmune mice after LCM challenge is attributed to the phenomenon described by Rowe, who observed that irradiated mice challenged 1 and 4 days post irradiation with LCM virus did not succumb to the viral infection but, instead, became carriers. In mice challenged 56 days post irradiation, no beneficial effect of the irradiation treatment was observed (*11*).

In the present experiment, LCM challenge was administered on the 21st postirradiation day. Tests of the blood and brain from these surviving mice yielded LCM in all 6 trials (table 3, expt. group C) on the 13th and 14th days after challenge. Hyperimmune mice challenged at the same time with the same viral preparation yielded LCM in only 1 of 12 tests (table 3, expt. group B plus expt. group E). This contrast favors the view that the phenomenon described by Rowe accounted for the survival of the 4 nonimmune mice (table 2, expt. group C), since the persistence of LCM virus in the irradiated mice was one of his observations. The experiments reported here give a closer estimate of the time interval over which this protective effect extends than did the experiments of Rowe (*11*), since it was present at 21 days but not at 33 days post irradiation, as seen in a subsequent experiment.

Since the bone marrow failed to protect against irradiation deaths in 6 of 12 (table 2, expt. group C) and 11 of 12 (table 2, expt. group D) nonimmune recipient mice, a third experiment was undertaken.

Assuming that 21 days post irradiation might be close to the limit of time for the protection afforded by the prechallenge irradiation, we

TABLE 3.—Results of viremia test of mice surviving challenge in table 2

Expt. group	Immune status of		LCM virus recovered	
	Host	Donor	Viremia	Virus in brain
B	Hyperimmune	—	0 0 0	0 0 0
C	Nonimmune	Nonimmune	+ + +	+ + +
E	Hyperimmune	Nonimmune	0 0 0	+ 0 0

TABLE 4.—Hyperimmune and nonimmune (BALB/c × DBA/2)F_1 mice protected after lethal irradiation by inoculation of marrow from immune or hyperimmune hybrid donors

Expt. group	Number of mice	X ray	Immune status of		Challenged*
			Host	Donor	Died/Injected
A	11	—	Nonimmune	—	11/11
B	8	—	Hyperimmune	—	0/8
C	12	800 r	Nonimmune	Nonimmune	7/7
D	14	800 r	Nonimmune	Hyperimmune	10/10
E	17	800 r	Hyperimmune	Nonimmune	0/15
F	16	800 r	Hyperimmune	Hyperimmune	1/13
G	8	800 r	Nonimmune	—	0/0†
H	5	800 r	Hyperimmune	—	0/0†

*Challenged on 33d day.
†Died of acute irradiation damage.

delayed the viral challenge until the 33d postirradiation day. No evidence of the phenomenon described by Rowe was observed. All 7 irradiated nonimmune mice protected with marrow from nonimmune donors succumbed to LCM challenge (table 4, expt. group C).

Immune mice that were irradiated and protected by marrow inoculation from nonimmune donors *survived* the LCM challenge (table 4, expt. group E), while nonimmune mice protected by marrow from immune donors *succumbed* to the viral challenge (table 4, expt. group D).

Bone marrow from immunized mice protected 13 of 16 immune mice from irradiation death. Twelve of these 13 survivors withstood LCM challenge (table 4, expt. group F). Since all mice were tested and found immune before irradiation, the single death from LCM in this group would indicate that irradiation destroyed a previously existing immunity in this mouse. A similar failure to survive intracerebral challenge with LCM after irradiation and marrow protection was observed in 2 of 9 mice in the second experiment (table 2, expt. group E). In both these experiments all immunized, nonirradiated mice survived LCM challenge (table 2, expt. group B, and table 4, expt. group B).

Three mice from each group of survivors in the third experiment were tested for viremia 2 weeks after challenge. All tests were negative. All surviving mice were rechallenged 1 month after the initial postirradiation challenge. All mice survived the rechallenge, demonstrating a solid immunity lasting over 60 days post irradiation.

DISCUSSION

In these experiments the immune status of irradiation-induced chimeras was tested by use of a fully potent, living pathogen.

Under the existing experimental conditions, the immune status, as measured by infectious challenge, was a function of the host and not the transplanted tissue. It further appeared that irradiation occasionally caused a significant weakening of the pre-existing immunity as demon-

strated by the observation that 3 of 22 immunized mice succumbed to challenge after exposure to lethal irradiation and protection with marrow from immunized mice (table 2, expt. group E, plus table 4, expt. group F). In the majority of the mice a previously established immunity remained unimpaired and in no instance did marrow from an immune donor confer immunity on a previously nonimmune host, even when combined with inoculation of splenic tissue from an immune donor.

A variety of experiments reported heretofore have yielded conflicting evidence, some indicating that antibody formation was of host origin (*1*, *3*), others suggesting that potentiality for antibody formation was transferred by donor spleen but not by donor marrow (*4*). Still other evidence shows that this potentiality may be transferred with the donor marrow (*5*, *6*, *12–14*). The present experiments are unique in that they concern immunity as measured by response to challenge rather than by demonstrable humoral antibody production.

The virus of LCM can be made to persist in carrier mice, or it can be induced to confer a solid immunity. It does not invoke in mice the neutralizing antibodies usually characteristic of viral infections (*7*). The present experiments indicate that tissue immunity of the type operative in murine LCM, as distinguished from that resulting from demonstrable humoral antibodies, was a function of the host. They suggest that the immunity in the chimeras described here did not depend upon function of the lymphocytic and other cells originating in the transplanted marrow (*15*, *16*).

Hollingsworth suggested that the conflicting theories of host-versus-graft and graft-versus-host reactions might be explained by assuming that the host produced humoral antibodies and the graft produced tissue antibodies (*3*). The present experiments, *within the limitations of the system employed*, would refute this assumption. The observations of Chin and Silverman (*4*) would also seem to conflict with the assumption proposed by Hollingsworth. Although donor marrow is doubtless competent under certain conditions, the present experiments evidently represent one of the limitations to this competence, since donor marrow clearly did not displace a previously established host-immune response. The irradiation as used here had no discernible effect on pre-existing immunity except for the occasional instances presented in which individual mice lost their ability to withstand viral challenge.

In formulating a working hypothesis that might reconcile the divergent concepts of immunologic competence in the irradiation-induced chimeras, it is advisable to delineate the area to be covered by the hypothesis. Evidence suggesting an immune response of host origin is derived from experiments by Makinodan and coworkers (*1*, *17*), Hollingsworth (*3*), and the experiments reported here. A salient factor in these experiments is the exposure of the host to an antigen *before* irradiation and marrow inoculation. Recent experiments of Chin and Silverman (*4*) demonstrate the transfer of the production of circulating antibodies by preimmunized donor spleen but not by preimmunized donor marrow to the chimeras.

Evidence for a response of marrow-graft origin is derived from several types of experiments, but in these the exposure to antigenic stimuli occurred *after* the irradiation or was evident from the use of sensitized donor mice: (*a*) genetic experiments, which first suggested the presence of immunologically competent grafts (*5, 12, 13*); (*b*) tests of the differential ability to respond to a particular antigen, by La Via *et al.* (*6*), which demonstrated an immune response of a rabbit fetal-tissue graft in the irradiated rat; (*c*) experiments with preimmunized donor mice (*10*), which gave further evidence that an immunologically competent graft may be demonstrated in these mice.

Depending on which set of data is considered, immune response may be of either host origin (*1, 3, 17*) or donor origin (*4–6, 10–14*). The following hypothesis is proposed as a possible way to reconcile these divergent concepts of host-graft interaction:

1. An antigen introduced *before* irradiation establishes within the host a pattern of immunologic response which is usually, though not invariably, radiation-resistant.

2. The irradiation may fix the immune mechanisms of the host to the conditions existing at the time of exposure. After irradiation, the host tissue responds with immunologic competence only to those antigens with which it had preirradiation experience.

3. The host does not respond immunologically to new antigenic stimuli or to stimuli to which it would not normally respond. Such a host would not react immunologically to a marrow homograft, unless it has been preimmunized to donor-strain antigenic material. Irradiation would usually not destroy the pre-existing ability to respond immunologically to the marrow graft.

4. Under conditions in which immunologic competence of the marrow graft exists, the graft may react against the foreign antigens of the irradiated host producing "secondary disease."

5. Such marrow may also impose on the host its own immunologic mechanism, as in the transfer of antibody production capabilities of the rabbit to the treated rat, in the development of the heterologous chimeras (*6*).

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Localization of Radioiodinated Antibodies in Rats Bearing Tumors Induced by *N*-2-Fluorenylacetamide [1, 2, 3]

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SUMMARY

Liver tumors, induced in rats by *N*-2-fluorenylacetamide (*N*-2-FAA), localized antibodies which had been prepared in rabbits against like tumors. The localization was followed by radioiodinating the antibodies and injecting them intravenously. These tumors also localized antibodies against normal liver. They did not, as a rule, localize antifibrin antibodies or fibrinogen during the 18 hours between injection and assay. In general they competed well with normal organs, such as liver, for circulating antibodies. In all these respects and by the ease with which they could be perfused, liver tumors induced by *N*-2-FAA sharply contrasted with transplanted tumors. In addition, radiolabeled antibodies which had localized in such induced tumors could be recovered from them by elution and returned to like tumors by the intravenous route.—J. Nat. Cancer Inst. 25: 787–802, 1960.

OUR OBJECT in this series of experiments was to determine whether autochthonous rat tumors differed from transplanted rat tumors in their ability to fix antibodies *in vivo*. We had shown previously that antibodies prepared in rabbits against several transplanted rat tumors localized in those transplanted tumors but that the localizing activity was due to antibodies which, by virtue of their reaction with fibrinogen in the circulation, were carried to the transplants during stages of rapid growth and/or inflammation (*1*, *2*).

Tumors were induced in rats by *N*-2-fluorenylacetamide (*N*-2-FAA), an agent well-known for inducing a variety of tumors at a variety of sites (*3–10*). Antibodies were prepared in rabbits against liver tumors induced by *N*-2-FAA, labeled with I^{131}, and purified from additional portions of tumor. They were then mixed with I^{133}-labeled rabbit normal serum globulin as a paired label control (*11*) and injected intravenously

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into rats bearing induced liver tumors. Zones of localization of antibody were determined 18 hours later.

Our results show clearly that induced autochthonous rat liver tumors behave quite differently from transplanted tumors. They are easily perfused—evidence in itself that such growths contain a well-developed, undamaged vasculature. They do not as a rule localize fibrinogen or antibodies purified from rat fibrin but do, on the other hand, compete effectively with normal organs and tissues in localizing antibodies prepared against themselves and against normal tissue.

MATERIALS AND METHODS

Production of Induced Tumors

Male Sprague-Dawley rats of the Charles River CD strain were used. The following procedure was adapted for the routine preparation of a pelleted, dust-free food containing a uniform distribution of *N*-2-FAA. Many of the steps were incorporated as safety measures to prevent contamination of the air with carcinogenic dust during food preparation, feeding of rats, and cleaning of cages.

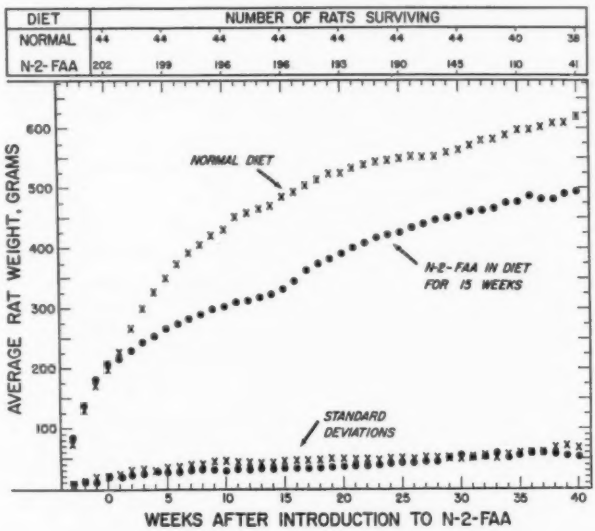
From a stock solution of *N*-2-FAA (Eastman Kodak) in acetone (25 gm./liter), 36 ml. (0.9 gm.) is mixed with 100 ml. cottonseed oil, heated to 120° C. to remove the acetone, and then slowly and thoroughly blended with 5 pounds of Purina laboratory chow meal in a closed Patterson-Kelley V-type liquid-solid blender. Immediately thereafter, 50 ml. of pure hot cottonseed oil is added to rinse any remaining compound from the high-speed liquid-feed bar assembly into the feed, and 2500 ml. of hot tap water (50° C.) is added to produce a uniform, stiff, dust-free, wet mash. The mash is transferred to the chamber of a sausage stuffer and worked into long bars ½-inch in diameter. These are cut into 1-inch pieces and dried at 60° C. in a forced-air drying oven for 18 hours to form solid pellets which are dust-free and easy to use as feed with a minimum of waste. The food, after drying, contains 0.036 percent *N*-2-FAA and 5.5 percent cottonseed oil. The average pellet consumption per rat per day is 20 gm. (corrected for waste), which includes 7.2 mg. *N*-2-FAA. The diet is available *ad libitum* for 15 weeks, during which time each rat consumes approximately 750 mg. of *N*-2-FAA. At all other times the rats are fed a normal diet, *ad libitum*, of Purina laboratory chow pellets. Water is available at all times. At any one time, 15 groups of 15 to 20 rats each are on the carcinogenic regimen. They are housed in cages with screen floors.

The rat colony from which we obtained tumor bearers for weekly assays contained at any one time 45 experimental groups of male rats started at weekly intervals. The newly arrived rats, averaging 80 gm. in weight, were kept on a normal diet for 3 weeks, at which time their average weight reached 200 gm. They were then given the carcinogenic diet (time zero) for 15 weeks, during which time their average weight increased to 330

gm., and again placed on a normal diet. Controls of the same age, on a normal diet, averaged 490 gm. at this point. After 26 weeks from time zero, some tumors began to appear, and from this time on, but usually between the 35th and 40th weeks, rats were used for assays and sources of tumor tissue for antibody production and purification.

Text-figure 1 charts the average weight gains of the first 12 experimental groups and the first 4 control groups. Usually, no weight loss occurred during the change from a normal to a carcinogenic diet, though the rate of gain decreased, which parallels the growth-response curves charted by Wilson *et al.* (*3*) for 50 gm. female rats. In general, the response of our rats (750 animals to date) has been similar to that reported by Miller *et al.* (*12*) for 180 to 230 gm. Sprague-Dawley males of the Holtzman strain, which gained 98 gm. in 16 weeks on a grain diet containing 0.03 percent *N*-2-FAA. Our 200 gm. rats gained 130 gm. in the 15 weeks on the carcinogenic diet and 290 gm. on the normal diet. Our observations contrast with those of Morris *et al.*, who reported (*9*) that male Buffalo strain rats weighing 195 gm. showed no gain after ingesting 580 mg. *N*-2-FAA for 23 weeks, and at 16 weeks weighed only 150 gm. However, controls from that strain, whose initial weight was 160 gm., gained only 140 gm. during 36 weeks. Laird and Barton (*13*) have also reported loss of body weight in Holtzman rats that started at 180 to 200 gm.

The high incidence of liver tumors in male rats on a diet of *N*-2-FAA is well established (*4–9, 12, 14–16*). Almost 100 percent incidence in



TEXT-FIGURE 1.—Average weight of 12 experimental groups of rats and 4 control groups as a function of time after initial exposure to *N*-2-fluorenylacetamide. The decrease in the number of rats in both experimental and control groups was caused mainly by withdrawals for assay purposes.

35 to 40 weeks is not unusual and has been our experience in these studies. We have been guided in histological identification of the tumors by the work of Edwards and White (*17*) and Firminger *et al.* (*18, 19*), and have adapted the Edwards-White nomenclature for our descriptions. The experience of others, such as Morris *et al.* (*20–22*), in distinguishing between mere hyperplasia and actual neoplasia has also been of value. We have necessarily been dependent upon gross examination for selection of tumors for assay, but one of us (J. P.) later examined specimens of each tumor by standard histopathological procedures for final diagnosis.[4]

We have observed, as did Laird and Barton (*13*), that although the extremes of liver neoplasia are either a single tumor mass easily distinguished and excised or a multitude of nodules which populate the whole liver and cannot be separated. The tumor nodules are usually about 0.5 to 1.0 gm. in size, 5 to 10 in number, and easily separated from the surrounding liver, an ideal situation for our work.

Preparation of Tissue Sediments

Tissue sediments have been described briefly in previous reports (*23*), but because of their importance as antigens for antibody production and as insoluble media for the purification of antibody, they are now described in detail. Fresh perfused tissue is homogenized at high speed in an Omnimixer in a medium of 5 volumes of ice-cold 0.9 percent NaCl buffered to *p*H 8 with borate. The homogenate is centrifuged at 2000 × g for 20 minutes at 4° C., the supernatant fluid is decanted and discarded, and the crude sediment is rehomogenized in 10 times its volume of borate-buffered saline. Centrifugation at 2000 × g and rehomogenization are repeated until the supernatant fluids are clear (about 5 times for liver, 4 for kidney, 2 for spleen, 3 for liver tumor). The next step involves separation of dense sediment from lighter sediment. The homogenate is separated, by centrifugation at 200 × g for 5 minutes and then at 2000 × g for 10 minutes, into two layers sufficiently packed so that, when the supernatant fluid has been decanted, the top, lighter layer can be dislodged as a unit from the bottom, dense layer by a fine stream of buffered saline, which is played upon it, and discarded. The procedure is repeated once or twice. This is an important step, since the amount of contaminating light cellular material in liver is about 10 times that of the dense stromas. In tumor there is a larger proportion of dense stroma than in the liver.

Preparation of Antiserums, Antiserum Globulins, and Radioiodinated Globulins

These methods and protocols have all been presented previously (*1, 11, 23, 24*). Both antitumor and antiliver antiserums were prepared in groups of 4 rabbits against the dense tissue sediment by intraperitoneal

[4] We are grateful to Mr. Eugene Burke and Dr. Eugene Studenski, of the Department of Pathology, for checking our diagnoses.

injection thrice weekly for 3 weeks, followed by a rest of 1 week. Antiserums in each group were pooled before globulin separation.

Purification *In Vitro* and *In Vivo* of Radioiodinated Antibodies

For purification *in vitro* the double-elution method (*23*) was used exclusively: 20 mg. of radioiodinated antiserum globulin was absorbed for 1 hour at 37° C. by 40 mg. lyophilized tissue sediment. The radioactive protein, which was eluted by heating at 60° C. for 20 minutes, was reabsorbed on another 40 mg. portion of sediment, and radioactive protein was again recovered by elution. Yields of radioactive protein, in terms of the percentage of original radiolabeled globulin recovered in the eluates, were comparable to those previously obtained (*23*), ranging from 0.2 to 1.0 percent.

The method of purification *in vivo* had been utilized successfully by Pressman and Sherman (*25*, *26*). The procedure used here varied in some detail from the earlier one. Radioiodinated antiserum globulin, usually 2 mc. and 20 mg. in 16 ml. solution, is injected intravenously into 4 rats, 4 ml. per rat. The next day these rats are perfused and their livers and liver tumors excised. Dense sediments are then prepared from the liver and tumor portions and suspended in 10 ml. borate-buffered saline. The radioactive protein is eluted by heating at 60° C. for 20 minutes. The eluates are injected and assayed in the same way as those obtained by purification *in vitro*. The two methods of purification, in fact, differ in only two respects: (1) *In vitro*, sediment is prepared first and then antibody is absorbed by it, whereas, *in vivo*, the antibody is first absorbed and then the sediment is prepared; (2) *in vitro*, a double purification procedure is employed and *in vivo*, a single.

Conditions for Assay of Radioiodinated Globulin in Rats Bearing Induced Tumors

The background localization of radioiodinated normal-serum γ-globulin in rats bearing autochthonous tumors induced with *N*-2-FAA was determined first. Table 1 presents a comparison of backgrounds of intrahepatic tumors and liver in 28 rats 1 day after injection. The tumors were typical of those later used in our antibody assays. Since the localizations per gram for liver and tumor were nearly mutually exclusive, it was apparent that the tumors had been separated from the liver with little trouble. The higher background in tumor than in liver, per gram, appeared to be related to greater concentration of stroma in tumor—three times as much stroma (in the form of dense sediment) was obtainable from tumor than from an equivalent amount of liver. The greater variability of the background in tumor may be related to greater variability in the stromal content of tumors—relatively low in type I hepatomas, moderate in type II, and high in adenocarcinomas (*17–19*).

The data in table 2 show the background localizations of radioactive normal globulin in 4 rats bearing transplanted tumors as well as 3 types

TABLE 1.—Nonspecific background localization of radioiodinated normal rabbit serum γ-globulin after 1 day in 28 rats bearing induced liver tumors

	Weight (*gm.*)			Percentage injected radioactive dose after 1 day			
				Total		Per gram	
	Rat	Liver	Tumor	Liver	Tumor	Liver	Tumor
Average	465	21. 9	6. 95	0. 96	1. 02	0. 044	0. 173
Standard deviation	56	4. 9	*	0. 33	†	0. 013	0. 097

* Tumor weights ranged from 1.68 to 17.2 gm. Twenty-three of 28 were in the 2 to 10 gm. range.
† The percentage of the injected dose in tumors ranged from 0.21 to 1.87.

TABLE 2.—Extent of nonspecific background localization of radioiodinated normal-serum γ-globulin in 4 rats bearing both transplanted and induced tumors

Tumor type and location	Weight of tumor	Percentage injected radioactive dose after 1 day in 4 rats	
	(*gm.*)	Total	Per gram
Transplanted Murphy lymphosarcoma in axillary region	11. 5	3. 8	0. 33*
Induced squamous-cell carcinoma in ear duct	7. 2	2. 2	0. 31
Induced hepatoma, type II,† intraperitoneal, extrahepatic	4. 9	1. 0	0. 21
Induced hepatoma, type II, intrahepatic	7. 5	0. 9	0. 12

* Average rat weight was 465 gm. In Murphy tumor of similar size in a 150 gm. rat, the total dose would be 6.9 percent and the dose per gram, 0.61 percent.
† Edwards-White nomenclature (*17*).

of autochthonous tumors induced by *N*-2-FAA. Whereas the induced hepatomas have a substantially lower background than the transplanted subcutaneous variety, the induced ear-duct tumors do not.

Table 3 shows the relative background localizations in tumor and liver, unperfused and perfused, 1 and 3 days after injection. Plasma is nearly as easily perfused from the autochthonous liver tumors as from liver. If they are assayed 3 days after injection rather than after 1 day, there is a further drop in background localization. In contrast, the transplanted tumor is not appreciably cleared of plasma either by perfusion (only 10% removed) or by extending the time of assay (only 10% decrease). It is obvious that the two most important features of the autochthonous liver tumors for assay of localizing antibodies are: greater perfusibility and lower background localization of nonspecific serum proteins.

RESULTS

Localization of Antibodies Purified *In Vitro* From Tissue Sediments

Table 4 shows that a substantial degree of localization in induced liver tumors was obtained with antibodies prepared against induced tumor sediment and purified from it. Although the liver fixed a much larger

TABLE 3.—Effect of perfusion and time of assay on nonspecific background localization of radioiodinated normal-rabbit serum γ-globulin in rats bearing induced liver tumors

Number of rats	Time between injection and assay (*days*)	Weight (*gm.*)			Percentage injected dose per gm. tissue				Ratio			
					Unperfused		Perfused		Unperfused/Perfused		Liver/Tumor	
		Rat	Liver*	Tumor*	Liver	Tumor	Liver	Tumor	Liver	Tumor	Unperfused	Perfused
4	1	458	26. 64	8. 82†	0. 179‡	0. 229‡	0. 041	0. 100	4. 7	2. 4	0. 91§	0. 53
4	3	515	30. 32	14. 67	0. 052	0. 166	0. 011	0. 086	4. 4	1. 9	0. 53	0. 24

*Perfused tissue weighs 20 percent more than unperfused tissue; weights of perfused tissue above are corrected for this; 30 percent of each liver was removed prior to perfusion to give the data on unperfused liver; 60 percent of each tumor was left unperfused.

†One of these tumors later was found to a metastatic lymphoblastoma with primary tumor in the spleen and metastases in both kidney and thymus.

‡The amount in humans, after 1 day, normalized to 500 gm. subject, would be 0.4 percent per gm. unperfused tissue (*27*).

§The ratio of human normal to tumor tissue is 0.92 ± 0.36 (*27*).

TABLE 4.—Net localization of purified antibodies in rats bearing induced liver tumors*

Purified rabbit anti-rat-tissue antibody†	Number of rats	Average weight (gm.)			Paired label member	Average percentage injected radioactive dose of purified antibody								
						Whole perfused tissue							Per gram	
		Rat	Tumor	Liver		Tumor	Liver	Kidney	Spleen	Lung	Heart	Thyroid	Tumor	Liver
Anti-induced tumor purified from induced tumor sediment	4	427	5. 66	25. 2	131	9. 12	20. 50	2. 08	1. 80	0. 49	0. 06	1. 7	1. 611	0. 892
					133	1. 73	1. 21	0. 11	0. 06	0. 34	0. 07	1. 9	0. 306	0. 048
					Net	*7. 39*	*19. 29*	*1. 97*	*1. 74*	*0. 15*	*−0. 01*	*−0. 2*	*1. 305*	*0. 844*
Antinormal rat liver purified from rat liver sediment	4	522	7. 70	24. 2	131	3. 44	23. 76	3. 74	2. 35	0. 51	0. 08	1. 5	0. 447	0. 984
					133	0. 91	0. 94	0. 13	0. 05	0. 28	0. 07	1. 3	0. 118	0. 039
					Net	*2. 53*	*22. 82*	*3. 61*	*2. 30*	*0. 23*	*0. 01*	*0. 2*	*0. 329*	*0. 945*
Antisolid transplanted Murphy lymphosarcoma purified from Murphy tumor sediment	6	404	11. 13	19. 5	131	4. 68	10. 42	0. 76	1. 52	0. 39	0. 05	2. 1	0. 420	0. 535
					133	1. 98	0. 60	0. 12	0. 08	0. 38	0. 05	2. 0	0. 178	0. 036
					Net	*2. 70*	*9. 82*	*0. 64*	*1. 44*	*0. 01*	*0. 00*	*0. 1*	*0. 242*	*0. 499*
Antirat fibrin purified from rat fibrin	6	474	4. 80	25. 0	131	1. 18	1. 15	0. 11	0. 14	0. 31	0. 04	1. 1	0. 246	0. 046
					133	1. 15	1. 16	0. 11	0. 07	0. 35	0. 05	1. 1	0. 240	0. 046
					Net	*0. 03*	*0. 01*	*0. 00*	*0. 07*	*−0. 04*	*−0. 01*	*0. 0*	*0. 006*	*0. 000*

*Purified I^{131} rabbit antiserum globulin minus I^{133} normal serum globulin.

†*See* p. 8 in text and table 5 in (#5) for description of purification procedure.

amount than did tumor, it fixed slightly less per gram. Antibodies prepared against normal rat liver and purified *in vitro* from liver sediment of normal rats also localized in the tumor and liver, but the amounts localized per gram were greater in the liver. Antibodies prepared against and purified from the dense sediment of the Murphy lymphosarcoma displayed the same general localization pattern, but to a lesser degree. In all three assays kidney and spleen also shared in the distribution of antibodies. Results with these antibodies contrasted sharply with those for antifibrin antibodies purified from rat fibrin. Whereas antifibrin antibodies in a rapidly growing transplanted tumor would have localized as much as 30 to 40 percent in induced tumors, we found no difference from the background localization of normal rabbit globulin.

Localization of Antibodies From Whole Globulin Fraction of Anti-Serums

The data in table 5 show that, with the use of the paired label, localizing antibody can be detected in the globulin fraction without any specific purification when induced liver tumors are used. This was barely possible in the transplanted tumors (*11*). The ability to demonstrate localizing activity without prior specific purification greatly simplifies the choice of serums and purification procedures. With whole globulin fractions, as with specifically purified antibody preparations, those antibodies that are prepared against tumor not only localize in tumor but also cross-localize in liver and the antibodies against normal liver not only localize in liver but also cross-localize in tumor. In normal rats, the anti-normal-liver antibodies localize in liver to a slightly greater extent than in tumor-bearing rats.

Extent of Localization of Radioiodinated Fibrinogen and Radioiodinated Antifibrin Antibody, Purified From Rat Fibrin, in a Variety of Induced Tumors

The tumors and normal tissues of the 6 rats, for which the assay of rat fibrinogen is given in table 6, show little if any localization above background, except in the tumor containing thrombi. As has been our observation in the past, the fibrinogen dose in lung and heart after perfusion was only a third that of normal globulin, while the dose in thyroid was half again as high and in kidney, slightly but consistently higher. It should be emphasized that the observed localization of fibrinogen is probably a good measure of the amount of fibrinogen accumulated in 1 day but does not necessarily represent the total amount of fibrinogen present.

The tumors and livers of the 6 rats, for which the assay of fibrin-purified antifibrin antibody is given in table 7, show the relationship between perfusibility and localization of antibody. In the first rat, which did not perfuse well, 26 percent of the dose was found in tumor and liver

TABLE 5.—Net localization of whole antiserum globulins in rats bearing induced tumors*

Whole rabbit anti-rat-tissue antiserum globulin	Number of rats	Average weight (gm.)			Paired label member	Average percentage injected radioactive dose of whole radioiodinated rabbit serum globulin*									
						In whole perfused tissue							Per gram		
		Rat	Tumor	Liver		Tumor	Liver	Kidney	Spleen	Lung	Heart	Thyroid	Tumor	Liver	Blood
Anti-induced tumor, pool No. 1	4	452	8. 46	20. 5	131	3. 41	2. 85	0. 35	0. 34	0. 36	0. 05	2. 0	0. 403	0. 140	1. 59
					133	2. 54	1. 08	0. 14	0. 11	0. 31	0. 04	2. 4	0. 300	0. 052	1. 59
					Net	*0. 87*	*1. 77*	*0. 21*	*0. 23*	*0. 05*	*0. 01*	*−0. 4*	*0. 103*	*0. 088*	*0. 00*
Anti-induced tumor, pool No. 2	4	485	9. 01	25. 4	131	2. 87	3. 66	0. 43	0. 26	0. 35	0. 05	1. 6	0. 318	0. 147	1. 24
					133	2. 11	1. 23	0. 13	0. 06	0. 28	0. 04	1. 7	0. 237	0. 050	1. 16
					Net	*0. 76*	*2. 43*	*0. 30*	*0. 20*	*0. 07*	*0. 01*	*−0. 1*	*0. 081*	*0. 097*	*0. 08*
Antinormal liver	6	472	7. 81	26. 7	131	1. 24	1. 65	0. 28	0. 12	0. 30	0. 04	2. 9	0. 158	0. 062	1. 16
					133	1. 05	1. 09	0. 13	0. 05	0. 29	0. 04	2. 5	0. 132	0. 041	1. 24
					Net	*0. 19*	*0. 56*	*0. 15*	*0. 07*	*0. 01*	*0. 00*	*0. 4*	*0. 026*	*0. 021*	*−0. 08*
Antinormal liver (in normal rats)	4	424	—	13. 7	131	—	1. 70	0. 39	0. 24	0. 43	0. 06	3. 2	—	0. 125	1. 61
					133	—	0. 86	0. 10	0. 08	0. 40	0. 05	2. 8	—	0. 063	1. 65
					Net	—	*0. 84*	*0. 29*	*0. 16*	*0. 03*	*0. 01*	*0. 4*	—	*0. 062*	*−0. 04*

*Whole I^{131} rabbit antiserum globulin minus whole I^{133} rabbit normal globulin.

TABLE 6.—Relative distribution of rat fibrinogen labeled with I^{133} and rabbit γ-globulin labeled with I^{131} in 6 rats bearing induced tumors

Number of rats*	Tissue type† and location	Perfused tissue weight (*gm.*)	Total		Per gram		Ratio
			I^{131} γ-globulin	I^{133} fibrinogen	I^{131} γ-globulin	I^{133} fibrinogen	Fibrinogen / Globulin
3	Hepatoma, type I (1) and type II (2), liver	8. 7	1. 243	1. 295	0. 14	0. 15	1. 04
5	Squamous-cell carcinoma, ear-duct	11. 3	4. 664	5. 380	0. 41	0. 48	1. 15
2	Adenocarcinoma, metastatic from liver, submaxillary	5. 5	1. 520	1. 010	0. 28	0. 18	0. 66
1	Adenocarcinoma, metastatic from liver, lung	8. 6	0. 987	0. 867	0. 12	0. 10	0. 88
1	Squamous-cell carcinoma, dorsal skin	0. 9	0. 393	0. 537	0. 45	0. 61	1. 36
1	Adenocarcinoma in liver (clotted blood present)	2. 8	0. 846	3. 257	0. 30	1. 15	3. 86
6	Remaining liver, normal	17. 7	0. 724	0. 694	0. 0410	0. 0394	0. 96
6	Normal kidney	—	0. 093	0. 107	—	—	1. 15
6	Normal spleen	—	0. 0497	0. 0484	—	—	0. 98
5	Normal lung	—	0. 244	0. 094	—	—	0. 39
6	Normal heart	—	0. 072	0. 022	—	—	0. 31
6	Normal thyroid	—	2. 23	3. 24	—	—	1. 45
6	Blood serum	—	1. 20	0. 15	—	—	0. 13
6	Unwashed blood clot	—	0. 11	1. 13	—	—	10. 3
6	Whole blood	1. 0	1. 31	1. 28	1. 31	1. 28	0. 98

*Average rat weight was 378 gm.; ear tumors were visible at 26 weeks, before many liver tumors had developed. Ear tumors were necrotic and hard to perfuse.
†Edwards-White nomenclature (*17*).

TABLE 7.—Variability in perfusibility of induced liver tumors and correlation with localization of I^{131}-labeled antirat-fibrin antibodies purified from rat fibrin and I^{133}-labeled normal rabbit globulin

Appearance of tissue after perfusion		Rat	Weight (*gm.*)		Percentage injected radioactive dose after 1 day in tissues (*per gm.*)					
					Liver		Tumor		Blood	
Liver	Tumor		Liver	Tumor	I^{131}	I^{133}	I^{131}	I^{133}	I^{131}	I^{133}
Bloody	Very bloody, many clots, nodular	510	22. 75	12. 30	0. 266	0. 194	1. 59	0. 542	0. 98	1. 33
Bloody	Quite bloody, nodular	602	21. 78	4. 60	0. 205	0. 186	1. 03	0. 500	1. 68	1. 70
Spotty	Spotty, nodular	621	20. 85	1. 04	0. 133	0. 127	0. 259	0. 254	2. 03	2. 10
Clear	Clear, nodular	505	18. 46	2. 74	0. 078	0. 078	0. 283	0. 290	2. 10	2. 07
Clear	Clear, diffuse	526	26. 40	In liver*	0. 044	0. 042	—	—	1. 81	1. 89
Clear	Clear, diffuse	515	38. 02	In liver*	0. 0182	0. 0187	—	—	2. 02	2. 03

*Examples of tumors which populate whole liver as minute nodules which are impossible to excise. Higher liver weights reflect this condition.

TABLE 8.—Localization in rats bearing induced tumors of radioiodinated anti-induced tumor antibodies purified *in vivo* from "tumor" and "normal" portions of livers containing induced tumors

Purified *in vivo* from	Average weight of 4 rats			Percentage injected radioactive dose of purified antibody*							
				Whole tissue						Per gm. tissue	
	Rat	Tumor	Liver	Tumor	Liver	Kidney	Spleen	Lung	Heart	Tumor	Liver
Tumor	578	3. 47	22. 34	5. 0	26. 8	2. 8	1. 8	0. 0	0. 0	1. 51	1. 25
Liver	549	3. 38	22. 61	3. 8	31. 8	2. 5	3. 3	0. 0	0. 0	1. 18	1. 42

* These are net values using as background-localization values 1.0 percent for whole liver, 0.2 percent for kidney, 0.1 percent for spleen, 0.4 percent for lung, 0.1 percent for heart, and 0.17 percent per gram of tumor.

combined; in the last rat, which did perfuse well, only 0.7 percent was found. Without the type of control that is possible with the paired label, there is danger of misinterpreting a high local concentration as antibody localization. For example, in the liver of the second rat the value for localization from the antibody preparation is about four to five times that expected for localization of normal globulin in a well-perfused liver; however, the high localization is due to poor perfusion, clearly shown by the fact that nearly as high a percentage of normal globulin localized as that of antibody.

Localization of Antibodies Purified *In Vivo* From Induced Liver Tumors

Radioiodinated anti-induced tumor antiserum globulin was injected in 4 tumor-bearing rats, and portions of radioactivity that were localized in tumor and liver were eluted the next day from sediments prepared from perfused tumor and liver tissue. The assay of the eluates in tumor-bearing rats is presented in table 8, which shows that the method of purification *in vivo* has selected antibodies which localize similarly to antibodies purified *in vitro* (*cf.* table 4). Considerably less is recovered *in vivo* (0.05% from 10 gm. of tumor, 0.2% from 90 gm. of liver) than by purification *in vitro* (0.6%), but the distributional differences are more evident. Radioactivity eluted from tumor localizes in higher concentration in tumor than in liver, while that eluted from liver localizes to a higher concentration in liver. These differences were noted consistently in each animal. Since extreme care was used in selecting animals for assay, even without use of the paired label the tumor and liver data stand out clearly. At present, the main value of this particular assay lies not in the slight differences it reveals between tumor-purified and liver-purified antitumor antibody but rather in demonstrating that antibody, whatever its specificity, localizes in certain induced tumors, can be purified from them, and can be returned to like tumors by the intravenous route.

DISCUSSION

When a radioiodinated antiserum globulin is injected intravenously into a rat, the distribution of the localizing antibodies contained in it is determined by the flow of blood to the organs and the efficiency with which they fix or trap the circulating antibody. This trapping efficiency is a function of the organs' content of accessible reacting antigens and the combining strength of the antigen-antibody combination (*28*). Rat kidney, for example, will localize two types of antibody: that directed against antigens accessible only in kidney and that directed against antigens which are common to many organs. The former will localize solely in kidney, and we call this specific localizing antikidney antibody. The latter will also localize in kidney but in competition with the other organs.

This we call cross-localizing antikidney antibody, that is, an antibody prepared against kidney but one with a potential for localizing elsewhere. In one such antikidney antiserum which we have reported, 51 percent of the kidney-localizing antibodies were found to be specific for kidney and 49 percent directed against antigens also accessible in other tissues to the circulation (*28*).

In transplanted rat tumors, despite many attempts, we were never able to demonstrate cross localization of antinormal tissue antibodies. On the other hand, antibodies against such tumors were shown to cross-localize extensively in normal tissues (*29*). The only specific type of localizing antibody shown to localize in transplanted tumors was antifibrin antibody (*1*), which, apparently by virtue of its combination with fibrinogen, was carried there during stages of rapid growth or extensive inflammation (*2*). The flow of blood to such transplanted tumors was so sluggish and the trapping efficiency so low that they presented no real competition to the other tissues and organs for cross localization. This situation in itself would appear to have made the detection of specific localizing antibodies much simpler by excluding the cross-reacting type. However, the extremely high background localization of imperfusible, nonspecific radiolabeled proteins in transplanted tumors prevented detection of small amounts of specific antitumor antibody. So far, purification prior to assay, in transplanted tumors, has netted only antibodies recognizable as those which cross-react with rat fibrin and which combine freely with fibrinogen (*1*, *30*).

In these experiments we have been able to show that certain induced rat tumors readily compete with normal organs, such as liver, kidney, and spleen, for circulating antibodies, for which they all have antigens more or less in common. Since the blood flow through such tumors is good, their efficiency in trapping circulating antibody is on a par with normal tissues.

The question of specificity of localizing antibodies has not been considered in these initial studies. We can be quite certain, however, that, since these induced tumors compete successfully for cross-localizing antibodies and exhibit low background localization, even small amounts of specific antibody should be demonstrable. It will be the object of our next series of experiments to look for such specific entities among the many possible types.

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Effect of a Transplanted Plasma-Cell Tumor on Antibody Formation [1]

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SUMMARY

Circulating antibody content after a single injection of sheep erythrocytes was observed in C3H/Lw mice bearing the plasma-cell tumor X5563. Hemolysin titers were within control limits for all mice tested whose tumors represented less than 5 percent of their body weight when they were killed. Among some mice, with tumors that weighed from about 5 to 30 percent of body weight, serum antibody content was lower with increased tumor weight, while in other mice bearing comparable tumors no serum hemolysin was detected even with a sensitive colorimetric procedure. The possibility of an increased rate of disappearance of autologous antibody was suggested by the sharp drop in serum hemolysin content at 5 to 7 days after antigen injection compared to that in controls, and from the observation in other experiments that passively transferred homologous antibody disappeared at a faster rate in tumor-bearing mice than in their controls. In addition, average blood volume in the tumor-bearing mice of one experiment was over 30 percent greater than in control mice of equal size and age. Hypervolemia would represent a dilution of the antibody if the primary response was equal and maximal in both tumor-bearing mice and their controls. Hemolysin titers were near control values in mice singly immunized after X-ray-induced regression of their plasma-cell tumors. A substantial secondary immune response was recorded in all mice tested with more than one injection of sheep erythrocytes or soluble antigens. These results suggest that the observed lowered antibody titers in the tumor-bearing mice after primary immunization may have been partly attributable to a reduction in the number of antibody-producing cells participating in the primary response.—J. Nat. Cancer Inst. 25: 803–812, 1960.

ABERRANT SERUM globulins are associated with multiple myeloma in human patients (*1*, *2*) and have been observed in mice bearing certain transmissible plasma-cell tumors (*3*, *4*). Increased susceptibility to infection and a reduced capacity to elaborate protective antibody specific to pneumococcus polysaccharides were reported clinically (*5*) in patients suffering from mutliple myeloma. An increased rate of disappearance

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of labeled, passively transferred antibody noted (*6*) among patients with advanced myeloma neoplasms suggests the possibility of abnormality in the metabolic degradation and synthesis of plasma proteins in the presence of the disease. These observations stimulate interest in experiments on antibody formation in animals with plasma-cell tumors, particularly since cells of the plasmacytic series are believed to be involved in immunity (*7*). Further impetus for such studies results from the availability of a plasma-cell tumor, transmissible in mice, which was described recently (*3*) and shown to resemble some human multiple myelomas in important respects. The experiments described here were carried out to study some physiological aspects of antibody formation in C3H mice bearing the transmissible plasma-cell neoplasm X5563.

MATERIALS AND METHODS

Tumor growth, blood volumes, and X-ray procedures.—Plasma-cell neoplasm X5563 was obtained in its 22d transfer generation and transplanted subcutaneously to C3H/Lw mice of both sexes, 3 to 4 months of age. Successful tumor growth was obtained in successive transplant generations during these experiments in 50 to 60 percent of the mice inoculated with pieces of the parent tumor by means of a 13-gauge trocar. The tumors grew equally well in both sexes, though wide variation in their growth rates was recorded among individual mice inoculated simultaneously. Solid tumors were dissected at autopsy and weighed after blotting.

Blood-volume measurements were made in some experiments from data obtained after the intra-arterial injection of Cr^{51}-labeled sodium chromate. Mice bearing plasma-cell tumors, or their controls, were lightly anesthetized with ether before exposing the left femoral artery, into which standardized amounts of a suspension of chromium-labeled isologous erythrocytes were transferred through a 27-gauge needle. The total red-cell volumes were estimated from the amount of radioactivity in initial and final samples of tail blood and corresponding hematocrit determinations, and from these values the blood volumes were calculated.

Antibody formation was studied in a few mice after their recovery owing to regression of the solid tumors by X radiation. Animals with relatively large tumors, placed in special holders providing appropriate body shielding, were irradiated with 2000 r of 200 kvp X rays and immunized 4 to 5 weeks after the tumors had become undetectable by palpation.

Serologic procedures.—Mice were immunized with intravenous injections of 2.5 percent suspensions of washed sheep erythrocytes. In some experiments immune response was studied after a single intravenous injection of 0.01 ml. of the cell suspension per gram body weight, while in other experiments the formation of antibody was studied when a second intravenous injection of the antigen had been made after an interval of 3 or 4 weeks. Serums were obtained from the femoral blood vessels at the time

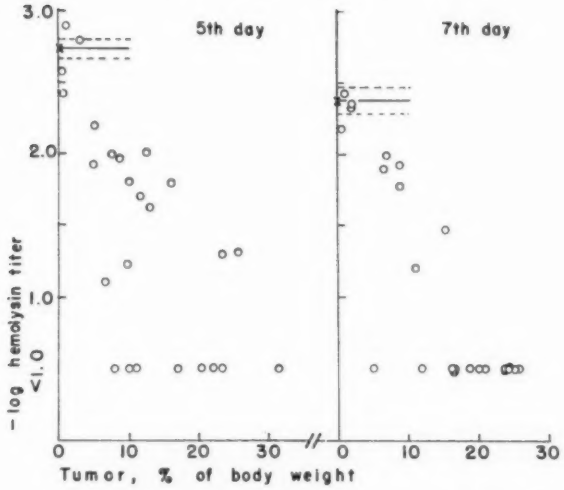
the mice were killed or from the tails when serial bleedings were done. Serum samples were heated at 56° C. for 20 minutes prior to freezing and storage in sealed plastic tubes at about −20° C. until the titrations were done, in most cases within 3 to 4 weeks. Serum hemolysin content was estimated colorimetrically by the procedure described by Taliaferro (*8*), and the titers were expressed as the negative logarithms of the serum dilution per cc. at which 50 percent hemolysis of a standard suspension of sheep erythrocytes was observed. Titers of log <-1.0 were too low for accurate measurement and were considered negligible. In a few experiments mice were immunized with soluble antigens. Five injections, intravenous alternating with intraperitoneal, of 5 percent solutions of bovine serum albumin (Armour & Co., Chicago, Ill., Lot #M12005) or 3 percent crystalline hen's egg albumin (Nutritional Biochemicals Corp., Cleveland, Ohio, Lot #4402) were given over a period of 3 weeks to test antibody formation with soluble antigens in mice with plasma-cell tumors. In these experiments the circulating antibody content was estimated by a modification of a hemagglutination procedure recently described (*9*).

Comparison of the disappearance rate of passively transferred antibody was made in control and tumor-bearing mice by serial tail-blood sampling after the intravenous injection of a 2× concentrated preparation of homologous antisheep-erythrocyte antiserum. This preparation was given in the amount of 0.03 ml. per gm. of body weight and the serum hemolysin titers were determined by the standard procedure already mentioned.

RESULTS

Hemolysin Response After Single Immunization

Previous experience with other strains of mice (*10*) and preliminary observations with C3H mice had shown that peak serum hemolysin titers occurred on the 5th day after single intravenous injections of sheep erythrocytes. Decreasing antibody titers were associated with increasingly larger X5563 tumors—in some mice 5 or 7 days after injection of antigen. Other mice appeared incapable of producing demonstrable amounts of serum hemolysin in the presence of relatively small tumors. Data for the hemolysin determinations on serums of individual mice are summarized in text-figure 1 and show that, with tumors weighing less than 5 percent of body weight, the circulating serum hemolysin values compared favorably with the averages for the control animals. Titers were <-1.0 for 23 of the mice tested at 5 and 7 days after antigen, even though the tumors in these mice ranged from 5 to about 30 percent of the body weight. Hemolysin titers in serums of the remaining 27 animals dropped progressively from near the average for controls to about −1.1 as tumor size increased. Correlation between tumor size and the amount of circulating abnormal serum globulin has been reported in mice with X5563 tumor (*4*). In a few cases not represented in text-figure 1, antigen



TEXT-FIGURE 1.—Serum hemolysin titers (O), 5 and 7 days after primary immunization with sheep erythrocytes, in C3H/Lw mice bearing the plasma-cell tumor X5563. Average titers (X) for non-tumor-bearing controls at 5 and 7 days (16 and 13 mice respectively) are shown with the 95 percent confidence limits.

was given directly into the tumor or intraperitoneally, with no improvement in the response.

No obvious correlation was found between the ages of the mice, length of time between tumor inoculation and antigen administration, and titers among the mice with negligible serum hemolysin. For example, of 3 mice 10, 11, and 17 weeks of age which were immunized at 4, 6, and 7 weeks, respectively, after tumor transplant, all had hemolysin titers <-1.0 when tested 5 days after antigen injection. Increase of the tumor masses in the mice of text-figure 1 averaged about 1.6 gm. per week between the 3d and 7th week after tumor inoculation. Tumor tissue, which may have been metastatic to other body regions, was not dissected at weighing but presumably influenced antibody formation. Immunological consequences of differences in growth rates of the tumors in different mice will be considered later in this paper.

Plasma-cell tumors were not associated with either a prolongation of the time required for the development or an earlier appearance of peak titers. The generally lower hemolysin titers observed in serums of tumor-bearing mice, 7 days after injection of antigen, are in accord with the drop in average titer for the controls at 5 to 7 days. In one experiment of this series, 7 mice bearing large tumors were bled from the tail on days 3, 5, and 7 after the antigen injection. One of these animals had a serum titer of -1.8 on day 3 and the remainder had <-1.0, while on day 5 one mouse had a titer of -1.95, while the remainder had <-1.0 for days 5 and 7. This result shows that among these mice, at least, there was no acceleration of the time for peak titer to be reached after

antigen was injected. Circulating autologous antibody may have disappeared more rapidly in the tumor-bearing mice (text-fig. 1) than in their controls because 32 percent (8 of 25) of the serums of the mice, 5 days after antigen injection, had titers of <-1.0, while 56 percent (13 of 23) of the 7-day mice had no detectable hemolysin compared to only a moderate drop in the control values at 5 to 7 days. Studies are in progress to determine the half life of autologous and heterologous antibody in mice bearing these plasma-cell tumors.

That serum from tumor-bearing mice did not significantly interfere with the hemolytic process *in vitro* during the titrations was shown by the result of the following experiment: A sample of the sheep-erythrocyte-mouse antiserum was divided into aliquots, each of which was then titrated by the standard method after dilution with equal volumes of (*a*) normal mouse serum or (*b*) serum from tumor-bearing mice. Resulting hemolysin titers for the antiserum were (*a*) -2.5 and (*b*), for 2 different tumor serums, -2.4 and -2.5. These results support the view that the depressed hemolysin titers in the tumor-bearing mice (text-fig. 1) are not due to technical difficulties or anticomplementary activity of tumor serum but may be attributed to physiological disturbances of the immune process.

Mice with plasma-cell tumors averaging 4.4 gm. received an intravenous injection of a concentrated sample of homologous sheep-erythrocyte antiserum. Hemolysin titers in 8 mice with tumors (hemolysin values in serum samples obtained 15 minutes after injection were taken as 100%) dropped to 70 percent at 24 hours compared to an average of 90 percent for 4 control mice. At 48 hours after injection of the antiserum, hemolysin had vanished from the serum of 2 and was about 50 percent of the initial value in 1 tumor-bearing mouse compared to an average of 70 percent for 3 control mice. This result is preliminary but indicates that passively transferred homologous antibody disappears more rapidly from the circulation in mice with X5563 tumors.

Blood Volume in Mice With Plasma-Cell Tumors

Observations made during blood sampling indicated the possibility that the mice with tumors had somewhat larger blood volumes than control animals. Table 1 summarizes the measurements in a few mice and shows that average blood volume was about 35 percent higher in 5 mice with large tumors than in 6 control mice of equal age and body weight. This result suggests the possibility that antibody titers observed for mice with plasma-cell tumors (text-fig. 1) may be too low by some factor represented by the excess in blood volume, if we assume that the primary immune response was equal and maximal in both tumor-bearing mice and their controls.

Effect of X-ray-Induced Tumor Regression on Hemolysin Response

Results summarized in table 2 show that antibody titers of 2 mice, O and T3, were close to the control value and somewhat lower than the con-

TABLE 1.—Average blood volumes in C3H/Lw mice with X5563 tumors and non-tumor-bearing controls

	A, 6 control mice	B, 5 mice with tumors*	Control/tumor
Calculated blood volume† ±standard error	1.9±0.1	2.9±0.3	0.65
Body weight, gm.	31.5±2.9	31.8±1.2	0.01
Blood in percent body weight	6.3±0.2	9.0±0.7	0.70
Hematocrit, percent	39.4±2.5	31.9±2.9	1.23

*Tumors ranged from 15 × 19 to 36 × 24 mm. No estimates were made for tumor volume.
†Blood volume was calculated from measurements made on the dilution of Cr^{51} tagged isologous erythrocytes after intra-arterial injection.

trol in 2 other mice, T2 and T5, after the irradiated tumors had regressed. Gross tumors in these mice decreased rapidly in size and had become unpalpable by 14 days after 2000 r tumor exposure. In 1 mouse of this experiment, T2, the tumor mass began to redevelop about 16 days after X irradiation. By 42 days the neoplasm had enlarged to about 9.1 $cm.^3$, at which time the mass was again exposed to 2000 r. The second treatment resulted in only moderate tumor regression and the mouse was immunized 10 days later. Extensive metastases to the spine and liver were observed at autopsy.

TABLE 2.—Hemolysin response in C3H/Lw mice after irradiation of the X5563 tumor

Mouse number	Tumor vol. at X irradiation, $cm.^3$*	Immunization		Hemolysin titer 5 days after antigen injection
		Days after X irradiation†	Tumor volume $cm.^3$	
O	11.8	51	Regressed	−3.0
T2	6.9	10‡	5.7	−1.9
T3	10.8	42	Regressed	−2.5
T5	8.8	42	Regressed	−2.0
Control, average of 16 mice				−2.7 ± 0.1 (S. E.)

*External measurement of the palpable tumors.
†Dose: 2000 r to tumor, body lead-shielded during exposure.
‡Tumor in this mouse had been irradiated twice as noted in text.

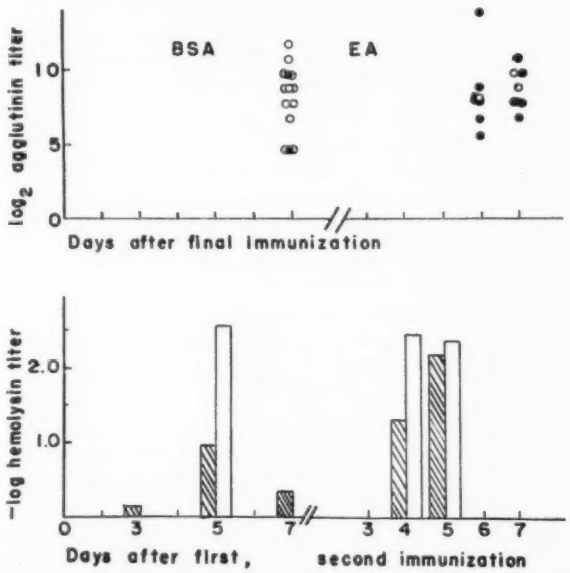
Immune Response After Multiple Immunizations

Preliminary observations of the hemolysin response in C3H and other strains of mice had demonstrated that, as was shown for rabbits (*11*), a second injection of sheep erythrocytes shortened the time required for the development of maximum titer. Peak hemolysin titers were observed in control mice 4 days after they received a second intravenous injection of sheep cells given from 7 to 21 days [4] after the first injection. Data from several experiments summarized in text-figure 2 show that a sub-

[4] Circulating hemolysin following the primary response has a half life of about 2.8 days in mice; thus, although not measured, the amount of antibody at the time of the second immunization was considered negligible.

stantial secondary response was elicited in mice bearing X5563 tumors, both with particulate (sheep erythrocytes) and soluble (bovine serum albumin, BSA, or egg albumin, EA) antigens. The *bar graph* (*lower part* of text-fig. 2) shows that the average hemolysin titer for 16 mice with tumors, 5 days after their first immunization, was −0.97 compared to −1.3 and −2.2 for 16 and 17 mice on the 4th and 5th days, respectively, after a second injection of the sheep-cell antigen. These results were confirmed on aliquots of some of the serums, with hemagglutination methods.[5] In the latter tests, titers were 4.8 ($\log_2$) for 5 mice and 8.9 for 10 mice at 7 days after the primary and 5 days after the secondary immunization, respectively.

Estimates of circulating antibody in serums after the last of several immunizing injections of BSA or EA shown for individual mice (*upper part* of text-fig. 2) indicate that mice bearing plasma-cell tumors were capable of responding to the soluble antigens almost as well as their non-tumor-bearing controls. Specific agglutinin was not demonstrable

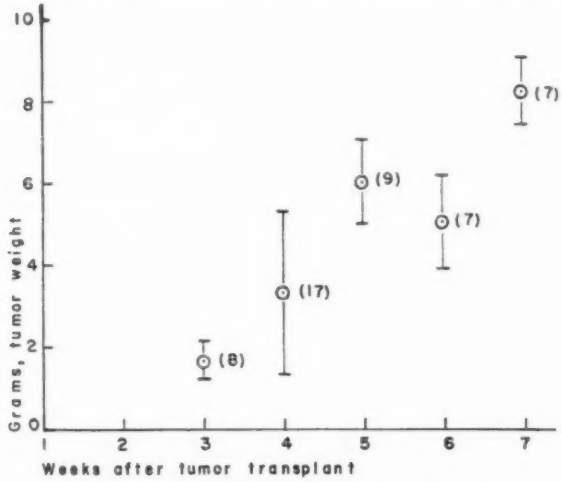


TEXT-FIGURE 2.—Secondary immune response in C3H/Lw mice immunized with particulate or soluble antigens. LOWER: *Shaded bars* represent average serum hemolysin titers in 10 to 20 mice with tumors at the indicated times after the first injection; at *right*, second injection of sheep erythrocytes given 7 to 21 days later. *Open bars* are average hemolysin titers in non-tumor-bearing animals. UPPER: Circulating antibody titers at indicated times after the end of immunization with either bovine serum albumin (BSA) or hen's egg albumin (EA) in mice with tumors (●) and their non-tumor-bearing controls (○).

[5] Twofold dilutions of the antiserum were incubated for 24 hours at room temperature in specially made tubes with 1 percent suspensions of fresh sheep erythrocytes. Antiserum dilutions in which the settling patterns most closely corresponded with similarly diluted serum from nonimmune mice were taken as the end points, and the $\log_2$ expresses the dilution of the antiserum.

by a modification of the recently described hemagglutination procedure (*9*) with formalinized sheep erythrocytes sensitized with BSA or EA, nor was precipitating antibody observed in the serums of the mice in these experiments after a single injection with the soluble antigen. Solid-tumor measurements made at the end of immunization in these mice ranged from about 24 × 26 mm. to about 43 × 30 mm., and immunization was completed by the 7th week after tumor transplant.

Lack of uniformity in the rate of growth of the tumors among different animals, suggested by the large error terms for tumor-weight averages (text-fig. 3), does not account for the fact that appreciable secondary immune responses were observed in mice receiving more than one antigen injection. All 17 mice with tumors had easily demonstrable amounts of hemolysin 5 days after a second injection of sheep erythrocytes (text-fig. 2), while over 40 percent of a comparable group of mice tested 5 and 7 days after a single antigen injection (text-fig. 1) had serum hemolysin titers of <-1.0. It therefore appears more likely that the X5563 neoplasms did not seriously interfere with the secondary immune response.



TEXT-FIGURE 3.—Average weights (○) in grams ± one standard error of X5563 tumors compared to time in weeks after subcutaneous transplantation in C3H/Lw mice. Numbers of mice per point given in parentheses.

DISCUSSION

Antibody titers were lower with increase in tumor weight after a single injection of sheep erythrocytes into some C3H/Lw mice with X5563 neoplasms, while in other mice bearing comparable tumors the primary response was undetected. Additional tests demonstrated a substantial response to a second injection of antigen, since antibody titers averaged

only slightly less than the titers observed in the non-tumor-bearing control mice similarly treated. These observations emphasize differences between the primary and the secondary processes of antibody formation. If it is assumed that relatively few competent cells participate in the primary response (*12*) and that these were reduced in number by the actively growing neoplasms, then correspondingly less circulating antibody would be formed and thus account for the lowered antibody titers observed (text-fig. 1). The apparent absence of a primary response in the other tumor-bearing mice (text-fig. 1) may have been the result of a still greater depletion of the small number of specialized antibody-producing cells. This process would reduce the amount of circulating antibody formed beyond the threshold necessary for its detection in the serum even with the highly sensitive serologic procedure used in these experiments. Observation of appreciable secondary responses among all the tumor-bearing mice tested supports the contention that the primary immune process was not abolished by the X5563 tumors.

Increased rate of metabolic degradation of the circulating autologous antibody by the cells of the neoplasm could also help to account for the lowered hemolysin titers seen in serums of singly immunized mice with tumors. This possibility is suggested by the preliminary observation that homologous antibody disappeared at a somewhat faster rate after passive transfer in tumor-bearing animals than in their controls. Relatively small amounts of antibody formed in some of the mice would have been diminished prior to serum sampling. In addition, the observed hypervolemia and consequent dilution of autologous antibody in the tumor-bearing mice may explain the lowered titers after primary immunization.

Evidence from the experiments just reported, showing that partial or complete recovery of the hemolysin response occurs after regression of the tumors by X irradiation, is in agreement with the temporary return to normal circulating globulins after surgical removal of large amounts of the plasma-cell neoplasms observed by Cobau *et al.* (*4*). The interaction between developing X5563 tumors and observed disturbances in globulin, including hemolysin, synthesis is reversible at least to the extent that either of the two treatments successfully removes the diseased tissue. Radiation-treatment studies are in progress with mice bearing X5563 tumors to determine the rate of recovery of the immune response after X irradiation of the neoplasm.

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Characterization by Certain Drugs of Four Morphologically Indistinguishable Cell Lines [1]

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SUMMARY

During investigations of cellular damage induced by various chemical agents, characteristic responses to certain of these were elicited in cell strains HeLa, D164, D189, and D227. The last three arose as a transformation of normal fibroblasts from infant's foreskin and are now morphologically indistinguishable. When hydrocortisone or Suramin sodium (naphuride sodium) was added to cultures of each cell line, these lines could be distinguished from each other by their differential responses as follows: Strain D227, after exposure for 1 to 2 days to 1 mg. per ml. Suramin sodium, showed fragmentation and beading of the cell membrane, cytoplasmic granularity, loss of spindle cells, and disruption of the typical reticulum-like pattern of early growth. The other three cell lines, when similarly exposed to Suramin at the same concentration, showed no response to this drug. Strain D189 exhibited marked cytoplasmic vacuolization after exposure to 0.01 mg. per ml. hydrocortisone for 3 days. Strain D164 exhibited moderate cytoplasmic vacuolization after exposure to a tenfold dose of hydrocortisone (0.1 mg. per ml.) for 10 days or more. Strain HeLa exhibited cell enlargement and nuclear aberration when similarly exposed to the same dose of hydrocortisone for 10 days. The typical responses of these morphologically identical cell lines demonstrate their pharmacologic distinctions. They also indicate the freedom from contamination of the D lines, among themselves and by strain HeLa.—J. Nat. Cancer Inst. 25: 813–825, 1960.

IN A previous communication (*1*), there was described a spontaneous transformation *in vitro* of a culture of normal human fibroblasts into cells which histologically resembled malignant cells. This cell line, designated strain D189, was not produced by deliberate cloning nor by subjecting it to any nutritional variations, which among other techniques may be responsible for producing cell variants (*2–8*). About the same time, a second culture of the same line of cells underwent a similar transformation and was named strain D164. A year later, a third culture exhibited analogous morphologic changes; it was called strain D227. These three

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[2] The authors are indebted to Mr. Walter G. Hardy for preparation of the photomicrographs used in this publication.

[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

converted cell lines thus arose from the same parent stem line of "normal" fibroblasts, derived originally from a fragment of infant's foreskin.[4]

As observed with transmitted-light and phase-contrast microscopes, the morphologic characteristics of the cells of these D lines are indistinguishable and very similar to those of strain HeLa and other strains grown in tissue culture. In all of them can be found three cell types: (1) a round, epithelium-like cell, (2) an elongated, spindle-shaped cell, (3) a polyhedral multinucleated giant cell.

In each of these cell lines, there is a lack of uniformity with regard to number, size, and position of the nuclei within the cytoplasm of the cells. The nucleoli may vary from 1 to as many as 8, their shape may range from polymorphous to spherical, and their size from small dots to large masses. Many combinations of these nucleolar characteristics can be found in a random assortment of cells within a cell population. Cell borders are well defined even when compact growth occurs.

In a program in this laboratory, which includes a study of cellular damage induced by a variety of chemical agents, some observations of special interest were made after exposure of the aforementioned D lines and of HeLa to Suramin sodium [5] and to hydrocortisone. This report describes the differences in response of these cells to the two drugs, differences which characterize the cells so that they can be readily distinguished from each other.

MATERIALS AND METHODS

Suramin sodium was dissolved in Eagle's medium at a concentration of 1 mg. per ml. The agent was sterilized by passage through a fritted-glass filter of ultrafine porosity.

Stock cultures of the cells were maintained in Eagle's medium in 2-ounce, narrow-mouth, half-oval prescription bottles. Suspensions were made by scraping the cells from the glass into 20 ml. of Eagle's medium. One ml. of this cell suspension was placed in a Leighton tube containing an 11 × 22 mm. coverslip. After incubation at 37° C. for 1 to 2 days, these cultures were ready for testing, and the medium in each tube was replaced with 1 ml. of fresh medium containing 1 mg. of drug.

Hydrocortisone [6] also was dissolved in the liquid nutrient and sterilized by filtration.

Cultures were exposed to 0.01 mg. per ml. or 0.1 mg. per ml. of this drug for varying periods, medium plus drug being replaced each day. The cultures were observed daily, ultimately fixed in Zenker's acetic solution, and stained with hematoxylin and eosin.

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[4] An account of the transformation of lines D164 and D227 will be published at a later date.

[5] Naphuride sodium (Winthrop Stearns, Inc., N.Y.) is the sodium salt of symmetric bis *m*-aminobenzoyl, *m,p*-methylbenzoyl-1-naphthylamino-4,6,8-trisulfonate. This drug was kindly supplied by Mr. Adrian Perrault.

[6] Solu-cortef, Upjohn Company, Kalamazoo, Mich., hereafter referred to as hydrocortisone.

RESULTS

The response of each of the four strains to the two drugs was distinctive. With Suramin sodium, strain D227 at the end of a 24- to 48-hour exposure to 1.0 mg. per ml. showed profound damage, while the other cell strains showed little or none.

The most significant changes in the cells of strain D227 were marked beading and disruption of the cell membrane, followed by fragmentation of the cytoplasm (*cf.* fig. 3 with fig. 1). These disruptive changes sometimes progressed to the point where the cells lost their nuclei and assumed a ghostlike appearance (fig. 2). In addition, and probably as a consequence of the cytological changes, cytoplasmic granularity, retraction of cytoplasmic processes, and occasionally, cellular blebbing were also seen. So pronounced were the nature and extent of the cellular changes produced by Suramin sodium in the D227 strain that one could readily determine the identity of this strain by its rapid response to this drug. In the other strains, the changes described appeared to a much lesser degree and only after several days of exposure to the same agent at similar concentrations.

A previous report (*9*) described the response of cells of the D189 strain to hydrocortisone, which produced cytoplasmic vacuoles that remained for several weeks. Figure 5 illustrates the pronounced vacuolization in cells of strain D189 after exposure to hydrocortisone at a concentration of 0.01 mg. per ml. for 3 days (*cf.* with untreated control cells in fig. 4). When the other three cell lines were similarly exposed, no such phenomenon resulted. Thus, this differential response to hydrocortisone could be used to characterize strain D189.

To distinguish between the two remaining strains, D164 and HeLa, the cells may be exposed to hydrocortisone at a concentration 10 times that used previously (0.1 mg. per ml.) for 10 days. At this concentration, vacuolization appears in sporadic groups of cells of D164 (*cf.* fig. 7 with fig. 6) but not in the HeLa cells (fig. 9). Thus, strain D164 can be distinguished. Although no vacuolization is observed in the HeLa cells, unlike the cells of D164, they may exhibit occasional cellular enlargement and nuclear aberration after 10 days' exposure to this high dose (*cf.* fig. 9 with fig. 8).

DISCUSSION

The differential responses of these four cell lines to the drugs used are significant for several reasons: (1) They demonstrate that no contamination of any of the D fibroblasts lines by HeLa has taken place and *vice versa*. In addition, cross contamination among the various D derivatives has not occurred, since each line behaves characteristically with hydrocortisone and Suramin sodium. (2) Further observations of the effects of the drugs on these cells also indicate that these D derivatives have become clearly divergent. They are now believed to represent com-

pletely different biological cell variants. Also, despite the histologic similarity of these strains and others in tissure culture (*10, 11*), there appears to be a characteristic difference in their rates and patterns of invasion of normal tissue (*12*). In addition, Dewitt *et al.* (*13*) have reported further dissimilarities of these four cell lines. These investigators found, when examining the relative frequency of chromocenters resembling sex chromatin, a higher frequency of these chromocenters in strain D189 (male) than in strain HeLa (female). On the other hand, strain D164 showed a low frequency of the chromocenters, and strain D227 was median between the other D derivatives.

The findings reported on the respective behavior of these lines toward the two agents, as well as those of other workers who employed the same cell strains, are consistent with the conclusion that the strains are now cytogenetically and physiologically dissimilar.

Established lines of human cells are now widely used for experimental purposes, and their utilization continues to increase. Because of their morphologic similarity, methods of distinguishing such cell lines from each other are urgently needed. Characterization of cell strains has been approached from many directions, such as nutritional and other biochemical requirements (*14–16*), chromosome counts and anomalies (*17–19*), sex-chromosome markers (*3*), morphologic variations (*8, 11, 12*), immunological responses (*20*), and virus susceptibility (*21*).

The drug responses of the cell lines used in this study offer an interesting possibility, that of devising a scheme for their identification.

For example, as shown in table 1, the one strain which responds markedly and rapidly to Suramin sodium after a 24- to 48-hour exposure is strain D227 (fig. 3). When all the strains are challenged with hydrocortisone for 3 days, only strain D189 yields a pronounced vacuolization response (fig. 5). Of the two remaining strains, D164 and HeLa, the former shows moderate cell vacuolization after prolonged exposure to a high dose of hydrocortisone (fig. 7) whereas strain HeLa cells do not. Finally, strain HeLa also responds in a different and characteristic manner when exposed to a high dose of hydrocortisone for 10 days (fig. 9) by exhibiting occasional cellular enlargement and nuclear aberrations.

This scheme for distinguishing the D derivatives and HeLa requires that the test be of relatively brief duration. Prolonged exposure (10 days) of the cells to either agent results in similar damage to all strains, with the exception of strain HeLa which does not respond typically to hydrocortisone even after this period of exposure.

One limitation of the procedure is that it is based on a comparative response of the cells to the drugs—it indicates *relative* sensitivities of the cells, *i.e.*, D227 is more sensitive to Suramin sodium than the other strains and D189 is more sensitive to hydrocortisone. Nevertheless, this procedure is shorter than many which are currently used to identify cell strains.

Note added in proof.—Since this paper was submitted, a reference appeared (Defendi, V. *et al.*: Immunological and karyological criteria

TABLE 1.—Responses to Suramin sodium and hydrocortisone of various human cell lines in tissue culture

	Suramin sodium			Hydrocortisone		
Cell strains	Dose (*mg./ml.*)	Time (*days*)	Effect	Dose (*mg./ml.*)	Time (*days*)	Effect
D227	1	1–2	Beading and fragmentation of cell membranes; retraction of cytoplasmic processes	0. 01	3	None
	—	—	—	0. 01	10	Moderate vacuolization
D189	1	1–2	None	0. 01	3	Marked vacuolization
	1	7	Beading and fragmentation of cell membranes	—	—	—
D164	1	1–2	None	0. 01	3	None
	1	7	Beading and fragmentation of cell membranes	0. 1	10	Moderate vacuolization
HeLa	1	1–2	None	0. 01	3	None
	1	7	Beading and fragmentation of cell membranes	0. 1	10	Cellular enlargement and nuclear aberration

for identification of cell lines. J. Nat. Cancer Inst. 25: 359–386, 1960) on the use of various criteria for identifying cell strains. Two of the cell lines used in our experiments, HeLa and D227, were found by these investigators to be immunologically and karyologically distinct.

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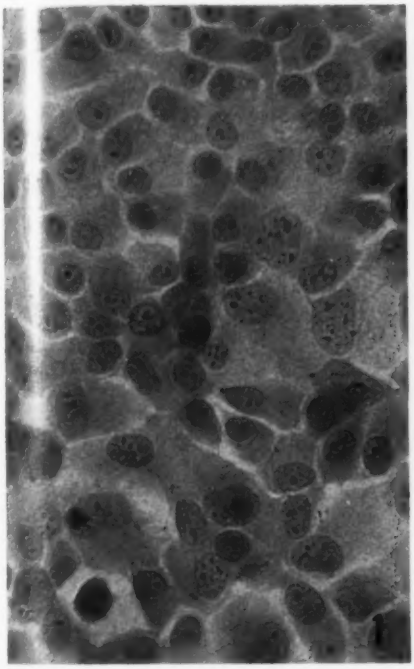
PLATE 112

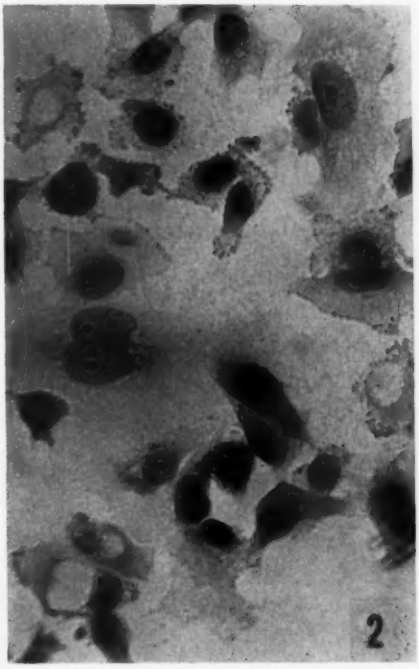
Effect of exposure to 1 mg. per ml. Suramin sodium for 2 days on strain D227-cells in tissue culture. Eagle's medium plus 10 percent human serum. Zenker-acetic fixation. Hematoxylin and eosin. × 360

FIGURE 1.—Untreated control cells. *Note* epithelium-like sheet of cells and well-defined cell outlines.

FIGURE 2.—Forty-eight hours after exposure. *Note* ghostlike cells with missing nuclei and motheaten appearance of cytoplasm.

FIGURE 3.—Forty-eight hours after exposure. Characteristic beading and disruption of cell membrane, fragmentation of cytoplasm, and cytoplasmic granularity. Cells are few in number, epithelial-type growth absent.





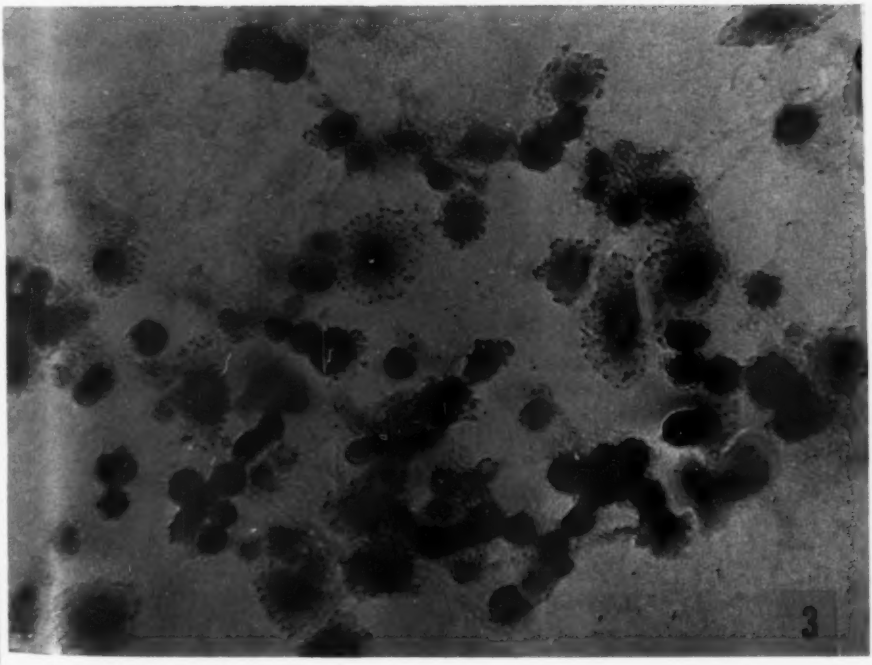
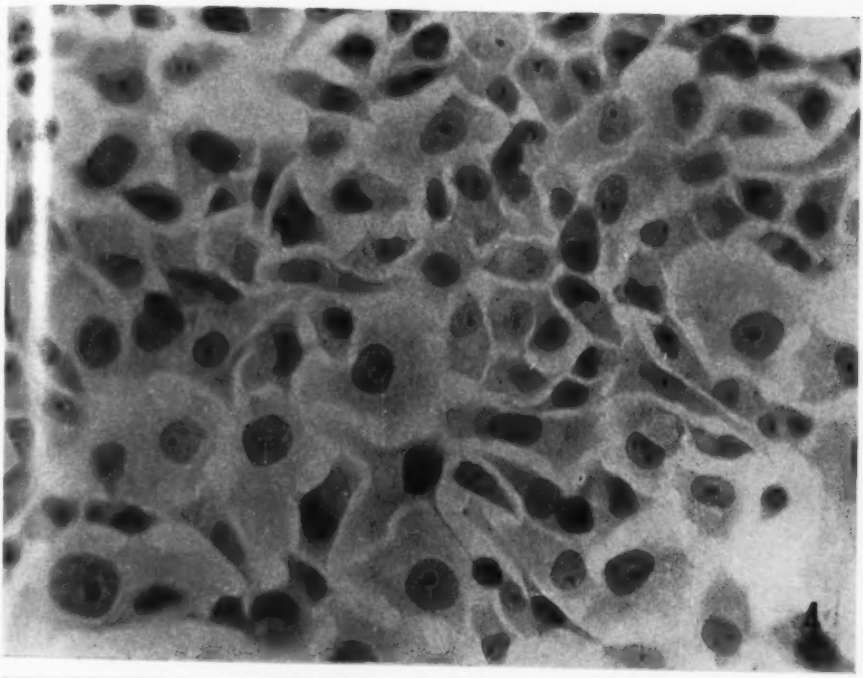


PLATE 113

Effect of exposure to 0.01 mg. per ml. hydrocortisone for 3 days on cells of strain D189 in tissue culture. Eagle's medium plus 10 percent human serum. Zenker-acetic fixation. Hematoxylin and eosin. × 360

FIGURE 4.—Untreated control cells. *Note* similarity to cells of line D227 in figure 1.

FIGURE 5.—Seventy-two hours after exposure. *Note* widespread and marked cytoplasmic vacuolization.



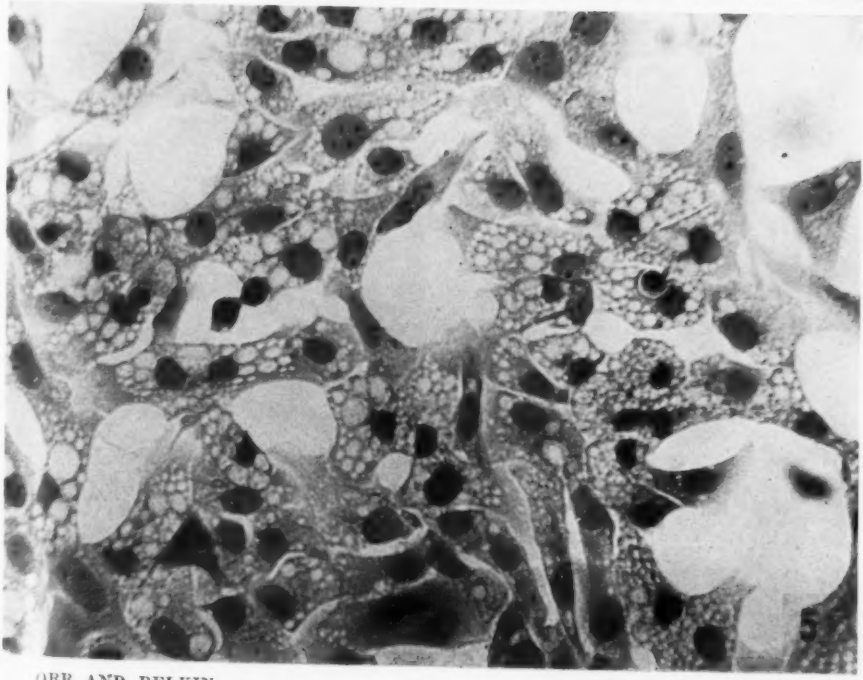


PLATE 114

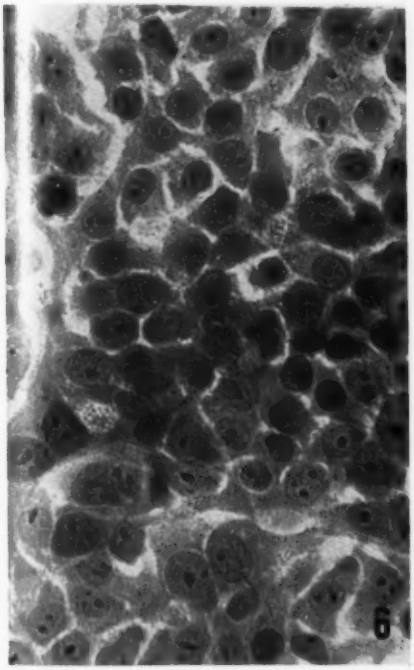
Effect of exposure to 0.1 mg. per ml. hydrocortisone for 10 days on cells of strains D164 and HeLa in tissue culture. Eagle's medium plus 10 percent human serum. Zenker-acetic fixation. Hematoxylin and eosin. × 360

FIGURE 6.—Strain D164. Untreated 10-day control cells. *Note* similarity to cells of strains D227 and D189.

FIGURE 7.—Strain D164 after 10 days' exposure. *Note* cell vacuolization, which is not so marked nor so widespread as in cells of D189 (fig. 5). Also, at this time, cell population density is diminishing.

FIGURE 8.—Strain HeLa. Untreated 10-day control cells. *Note* similarity of cells and pattern of growth to strains D227, D189, and D164.

FIGURE 9.—Strain HeLa after 10 days' exposure. *Note* cellular enlargement and nuclear aberrations.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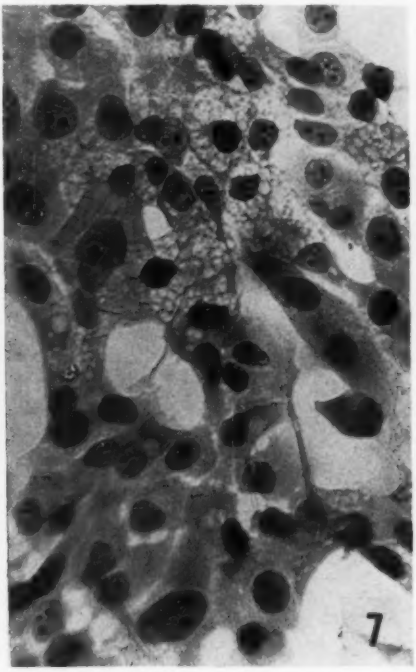
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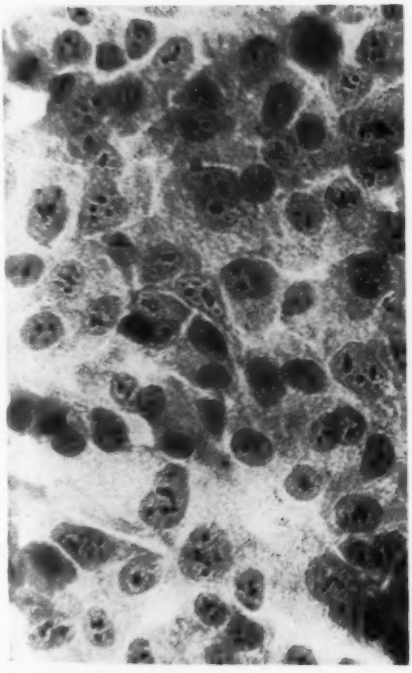

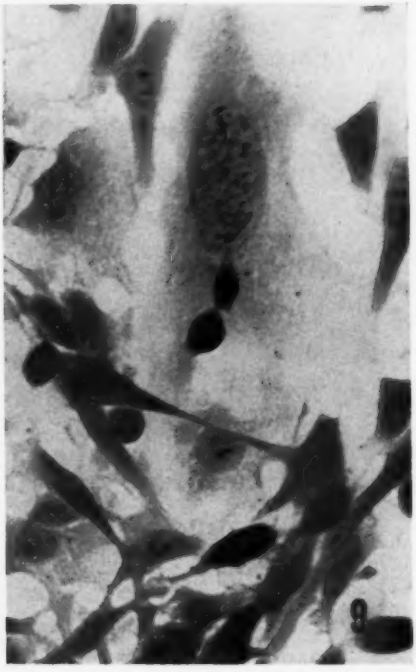
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An Electron Microscope Study of Spontaneous Mammary Carcinomas in a Subline of Strain DBA Mice [1, 2]

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SUMMARY

Spontaneous mammary carcinomas of DBA/212 mice proliferated milk-agent particles more abundantly than any tumors studied so far. Acinar spaces were usually filled with the characteristic 100 mμ particles containing nucleoids, and the cell membranes which border the spaces were lined with the forming particles in all stages of development. Particle formation appeared to be at the cell membrane which thickens on the inside and protrudes to form a hemisphere, then further thickens and forms a horseshoe-shaped spheroid, and finally a very thick-shelled sphere which pinches off from the cell. The dense material of the sphere then rearranged itself to form a nucleoid, 20 to 40 mμ in diameter, surrounded by a membranous sac which appeared to be the same morphologically as the cell membrane. While the particles were still attached to the cell they were remarkably constant in diameter, but in the acinar spaces many of them appeared swollen and some contained two nucleoids. Within the cytoplasm of these tumor cells there was an abundance of dense, thin-shelled 60 to 70 mμ spheres of type A, as described by Bernhard. The spheres were usually associated with cytoplasmic vacuoles, but were rarely found near the cell border. Except for these particles and vacuoles, the cells which were so actively exfoliating the milk agent appeared normal and healthy.—J. Nat. Cancer Inst. 25: 827–845, 1960.

THE PRESENCE of specific particles in thin sections of mouse mammary carcinomas was first revealed, by electron microscopy, in a transplantable tumor of a CBA mouse and reported in 1953 (*1*). An abundance of complex particulate structures had, however, already been observed, in unsectioned whole mounts of cultured explants from C3H mice, by Porter and Thompson (*2*). Since these early observations, a number of reports have described similar particles, which are thought to be the mammary-tumor agent, in mammary carcinomas of C3H and RIII mice (*3–6*). Bernhard *et al.* (*7, 8*) have described this agent in IC (Institut de Cancer) and T120 mice. More recently Ichikawa and Amano have found similar particles in a mammary tumor of strain SL mice (*9*). When Bittner

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first established the existence of the milk agent by foster nursing, he used an A strain which had a high incidence of mammary carcinoma (*10*). Bioassay has demonstrated the presence of the milk agent not only in many inbred mice but also in sporadic tumors of wild mice (*11*).

Bernhard, in an extensive review (*8*), lists the existing number of mouse strains having a high incidence of mammary tumors in which agent particles have been noted. No mention was made of the DBA strain. A search in the literature, however, revealed that a paper by Suzuki (*12*) makes a brief reference to the presence of the milk agent occurring in the tumors of strain DBA mice, but gives no detailed description or illustration of this agent or of the specific subline of the DBA strain used.

This paper describes in detail the presence of characteristic particles in the spontaneous tumors arising in a subline of DBA mice, and explains the process by which the agent is produced.

MATERIALS

The DBA strain of mice was originally established in 1909 by Dr. C. C. Little through selecting three recessive genes—dilute, brown, and non-agouti, from which the name of this strain is derived—for color experiments (*13*). This strain proved to contain genes promoting mammary-gland tumors. Many sublines have been developed from this original DBA strain which are being used in various research laboratories in the United States and abroad. The subline used in this study is designated DBA/212. Several breeding pairs of this subline were originally obtained by one of us (A.G.) from Dr. T. C. Hauschka, in 1948, and have been inbred by sister × brother mating since then. Spontaneous mammary tumors occur among females 7 to 18 months of age, the incidence being about 90 percent.

RESULTS

Type of Tumors

The spontaneous tumors arise mainly in the inguinal and axillary regions and in some cases multiple tumors appear in the same mouse. An example of such an instance is shown in figure 1. The tumors are usually round and firm and when they reach considerable size, frequently ulcerate.

Light Microscopy

The histological structure, as revealed by light microscopy (fig. 2), consists of aggregates of closely packed cells and some acinar spaces bordered by a single or double layer of columnar epithelium.

The cell nuclei are uniform in size but vary in shape, some appearing round or oval and others slipper-shaped or irregular. Nucleoli are promi-

nent, and occasionally mitotic figures are noted among the cellular aggregates.

Electron Microscopy

Specimens for electron microscopy were excised from the tumors of anesthetized mice, fixed immediately in buffered 1 percent osmium tetroxide containing 0.1 M sucrose, rinsed in 50 percent ethanol, rapidly dehydrated in 70, 95, and 100 percent ethanol, and embedded in deoxygenated methacrylate (*14*).

Sections were cut with a Porter-Blum microtome, stained with lead hydroxide according to the procedure of Watson (*15*), and photographed in an RCA EMU2 electron microscope.

Many sections from tumors of 8 different mice were examined and characteristic particles were observed in every section. However, only those cells which border an acinus seemed to be actively propagating the characteristic particles (*cf.* figs. 4 and 5). In figure 4, the cells border an intercellular space. The cell borders are covered with budding particles and the space is filled with particles containing nucleoids, which are regarded as the mature agent. Figure 5 shows parts of 4 cells with no intercellular space. These contain many vacuoles surrounded by dense ring-shaped particles measuring 60 to 70 mμ in diameter.[4] In this micrograph there are only a few of the characteristic nucleoid-containing particles, and they are in the vacuoles. In figures 3, 6, and 7, an abundance of both types of particles is seen. The smaller dense rings are within the cytoplasm and the larger particles with nucleoids are in the intercellular space. Any relation between the two types of particles is not evident here. Further details of the appearance of these particles and their propagation are illustrated in figure 6, and the essentially normal cytology of 2 cells is illustrated in figure 7. A comparative illustration is presented in figure 8 which shows cells of a normal lactating gland of a tumor-resistant female mouse of the C57BL strain. Milk particles in the interlobular duct vary in size and contain no internal structure.

The steps in the formation of the milk agent may be reconstructed from the electron micrographs. First, there is a protrusion of the cell membrane, with an accumulation of dense material on the inside and the formation of a hemispherical boss (*see* figs. 9 and 10). The hemisphere gradually turns into a sphere by completely filling in the proximal portion and a simultaneous over-all increase of density. After the particle separates from the membrane there is a rearrangement and possibly some loss of the dense material. The nucleoid is formed leaving a zone of lesser density between it and the membranous sac. At this time there is an increase in volume as evidenced by the slightly greater diameter of particles, which are some distance from the cell membrane, than that of those still connected to, or lying very near, the membrane.

[4] The particles are, of course, actually spheres. The variation in size and density of the profiles shown results mainly from the level at which they are sectioned. The section thickness is not known exactly but is probably somewhat less than one half the particle diameter.

Some of the particles in the intercellular spaces contain two nucleoids and some have greatly distended membranes, a few of these losing their spherical appearance (fig. 6). The over-all size of the more spherical particles ranges from 85 to about 180 mμ, but more than 90 percent of them have diameters between 95 and 110 mμ.

DISCUSSION

The concentration of mammary-tumor agent, as determined from thin sections, in these DBA/212 tumors seems greater than that of any other mice studied so far. Tumors from 8 different mice have been examined and the abundance of particles portrayed by the accompanying electron micrographs has been found in almost every section. In previous experiments (*16*), in which attempts were made to correlate biological activity with the specific structures as seen with the electron microscope, RIII mice were used as a source of the agent. In the tumors of this strain, although structures that are now considered as agent particles were seen as early as 1953, they were somewhat difficult to find until explants of these tumors were grown in tissue culture (*17*).

Although in the DBA/212 mice the characteristic particles can be seen in tumor areas that grossly appear necrotic, the cells in which the particles are propagating appear intact and in good condition; the nuclei, the endoplasmic reticulum, and the mitochondria all appear normal. The only indications of abnormality are the occurrence of vacuoles and possibly changes in the Golgi region, accompanied by the appearance of the dense ring-shaped bodies near the vacuoles or in the Golgi zone. These 60 to 70 mμ bodies with interiors of low density have been described in detail by Bernhard and collaborators (*7*, *18*) who designated them as type A bodies. These bodies are frequently seen in sections of tumors of the DBA/212 line, whereas they were seldom observed in tumors of RIII mice and never in explants from RIII tumors grown in tissue culture (*17*). However, the areas which contain these type A bodies have been correlated by Bernhard, Bauer, Guérin, and Oberling (*7*) with inclusions readily visible in the light microscope. These have been thoroughly studied by Guérin (*19*, *20*) and have been found in the mammary tumors of many strains of mice. They may represent a variant of the milk-agent bodies rather than a precursor of it. In our micrographs all stages of formation of B particles can be seen at the cell membrane, while groups of A particles remain in the perinuclear area of the cytoplasm. In some cells, *e.g.*, RIII tumor explants (*17*), only B particles were observed whereas in others predominantly A particles were seen, as shown in figure 5. In this DBA strain, however, most producing cells of the tumor seem to synthesize both A and B particles (figs. 3, 6, and 10), but there is no evidence that B particles are usually formed from A particles, although occasionally they may be. The significance of the A particles is not yet understood, but their association with cancer tissue is well established and they may prove to be an important agent in tumorigenesis.

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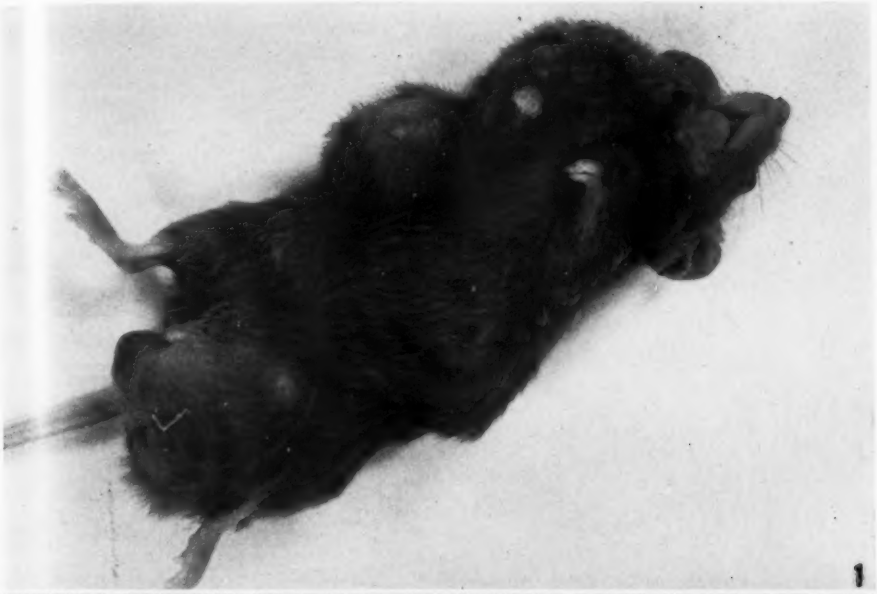
PLATE 115

FIGURE 1.—Female mouse of strain DBA/212, 16 months of age, showing 4 spontaneous tumors—3 large and 1 small.

FIGURE 2.—Light micrograph of tumor section. Field shows nests of closely packed cells surrounding numerous acinar spaces where milk-agent particles are usually found by electron microscopy. × 400



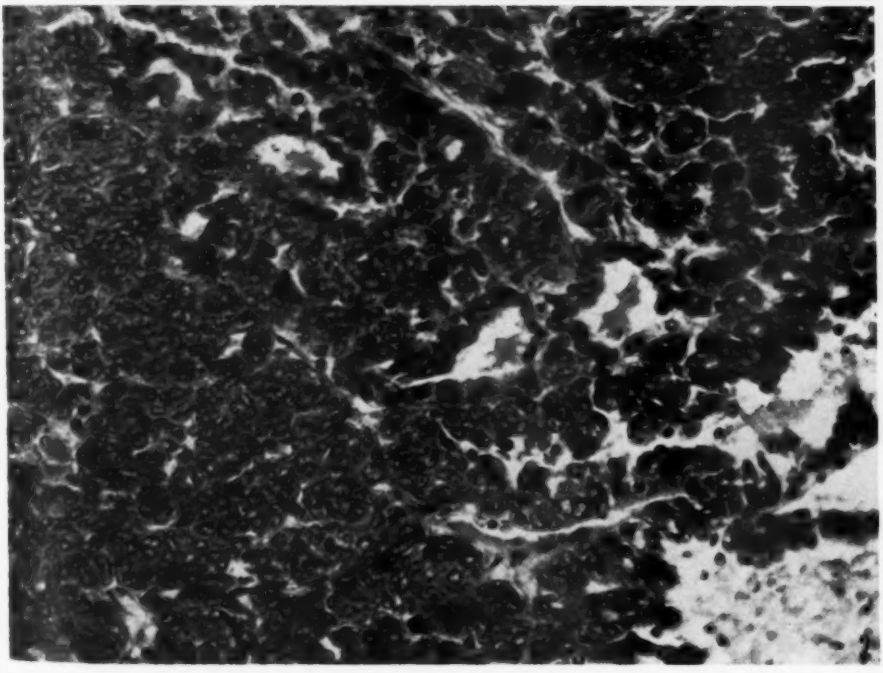


PLATE 116

FIGURE 3.—Portions of 2 tumor cells illustrating vacuoles surrounded by dense ring-shaped 70 mμ particles and at same time various stages in formation of the milk agent. An early step in its formation is believed to be represented at *a*, later stages at *b* and *c*, and the mature virus at *d*. Vacuoles bordered with dense particles are not believed to represent invaginations of the cell membrane from above or below the plane of section because these 70 mμ ring-shaped particles are never seen near cell surface. Vacuoles contain a few mature milk-agent particles of unknown origin. Occasionally, however, half-formed particles, as at *b*, have been observed that extend from vacuole membrane into its lumen. × 38,000

FIGURE 4.—Acinar space filled with milk-agent particles which apparently have been shed from membranes of bordering cells. × 28,000

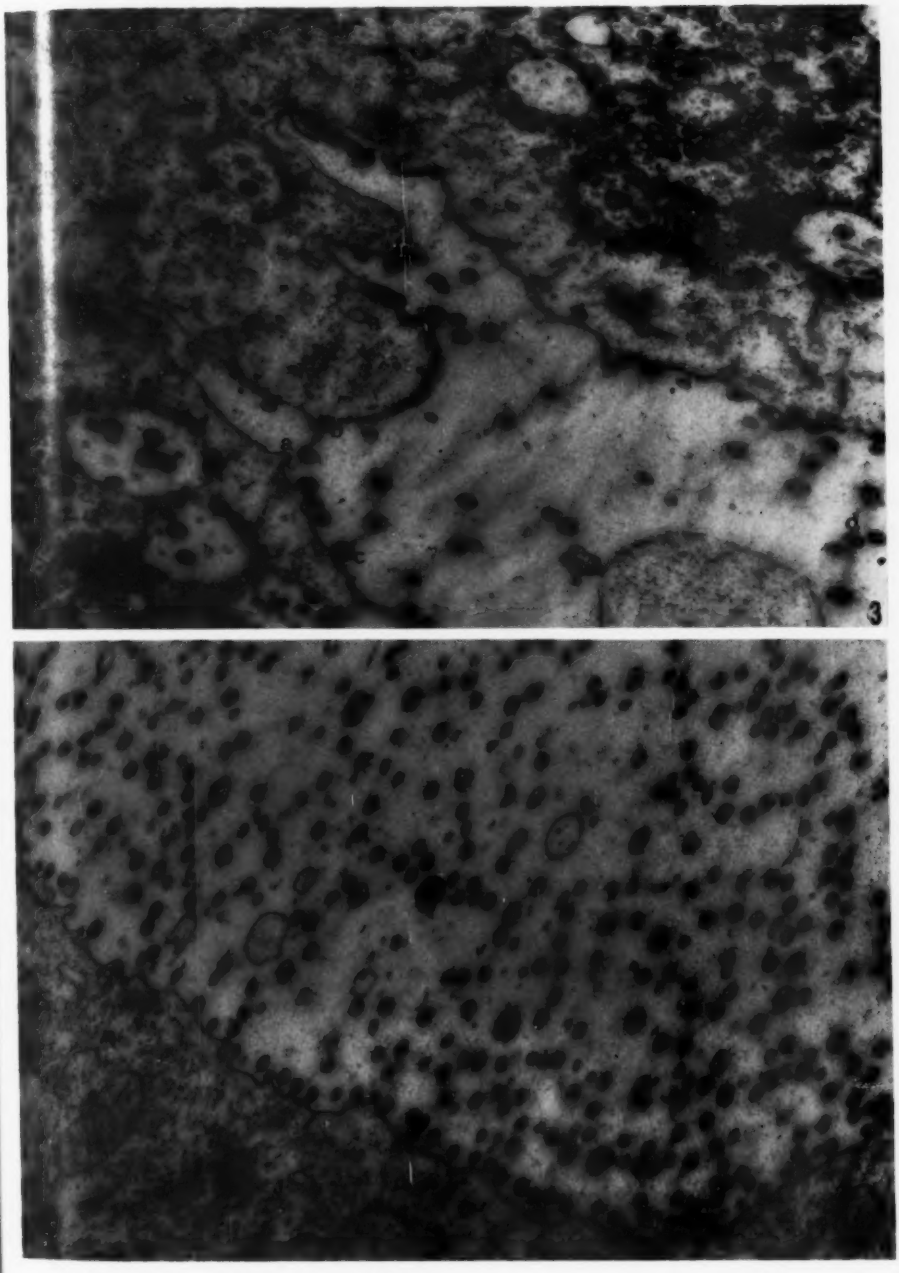


PLATE 117

FIGURE 5.—Section showing parts of 4 tumor cells with many vacuoles surrounded by doughnut-shaped, type A particles. There is no intracellular space and only a few recognizable milk-agent particles, these being within vacuoles. Membrane-enclosed vacuoles are in effect extracellular. × 38,000

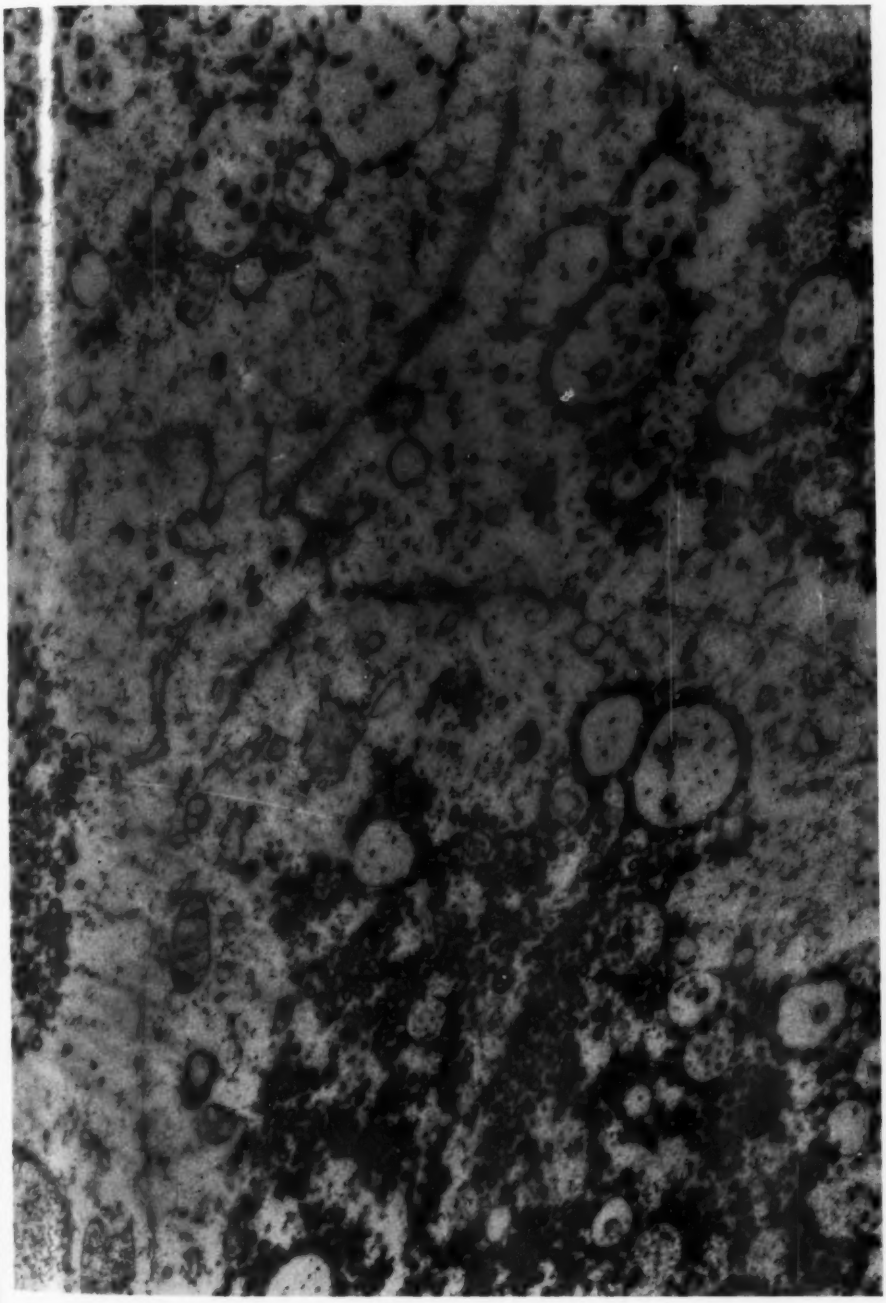


PLATE 118

FIGURE 6.—Section of mammary carcinoma showing an acinar space filled with milk-agent particles. Particles in various stages of formation can be seen along cell borders. Membrane thickens, protrudes as at *a*, and particle, at first quite dense, pinches off. Profiles of those which lie near cell membrane are doughnut-shaped, whereas those slightly removed from membrane contain distinct nucleoids which have separated from limiting sac. These particles are about 100 mμ in diameter, some are larger and some contain two nucleoids. Within cell (*left center*) is a group of smaller doughnut-shaped bodies 60 to 70 mμ in diameter. Many densely stained RNP granules can be seen in cytoplasm, both in contact with and free of endoplasmic reticulum. × 48,000

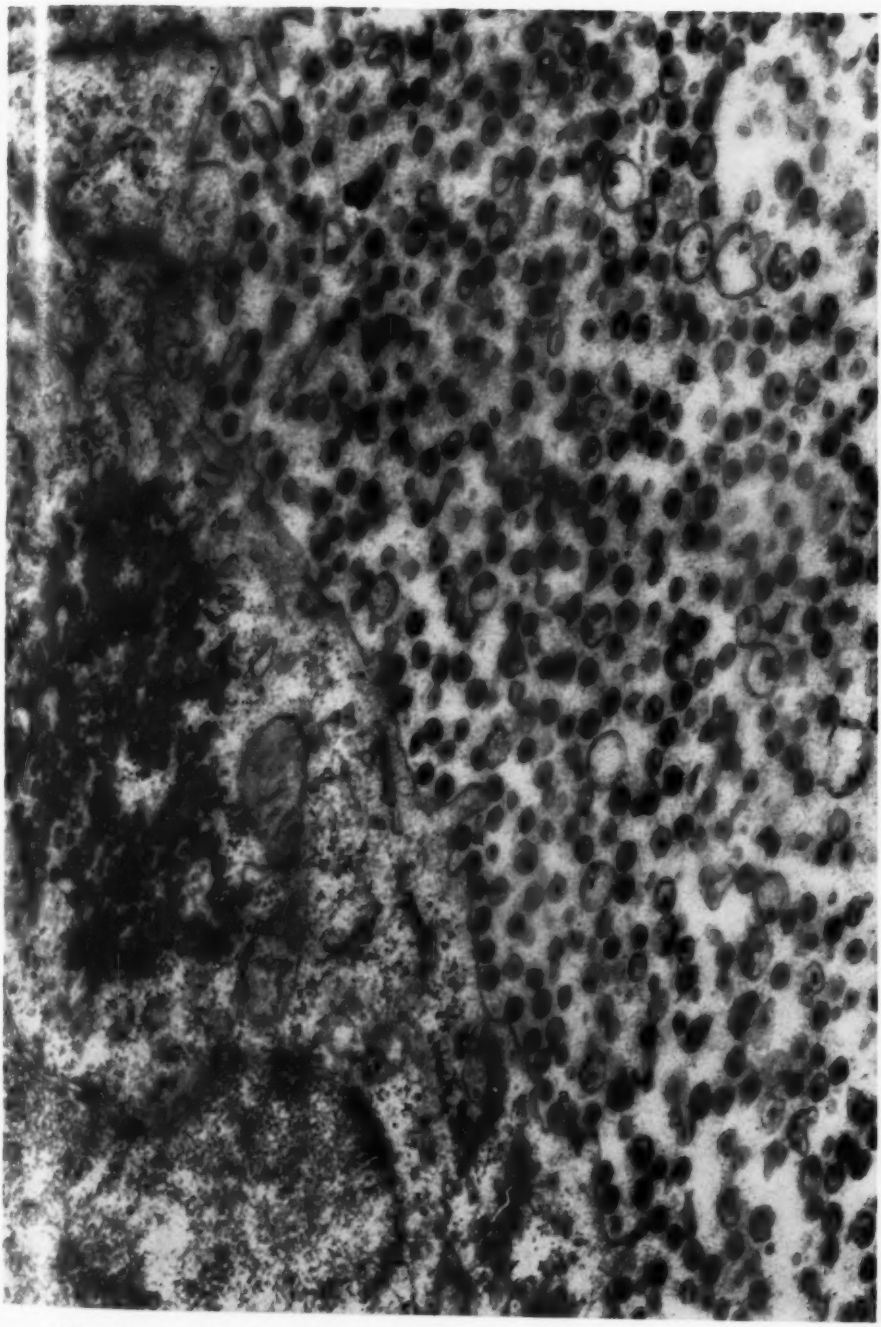


PLATE 119

FIGURE 7.—Area of tumor showing its apparent healthy state except for vacuoles and type A bodies of cells which may be regarded as source of milk agent. Nucleus, N, with double membrane, Golgi zone, G, mitochondria, m, and endoplasmic reticulum all appear normal. The very irregular border of acinus in *left lower half* of picture shows many milk-agent particles in various stages of formation. Microvilli are extending from above or below the plane of section in such a way that many particles are caught in their premature stage before they pinch off and form nucleoids. × 32,000

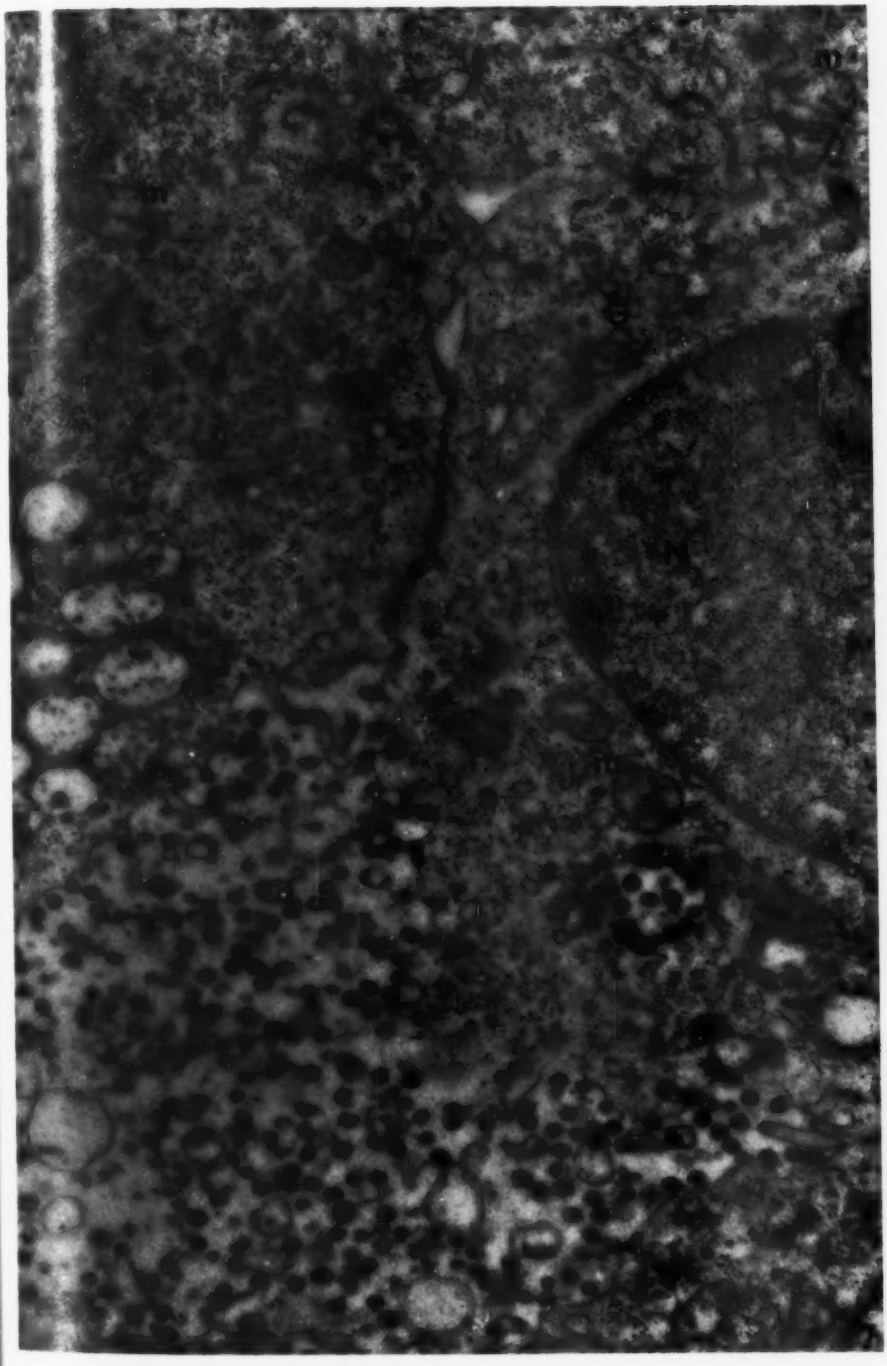


PLATE 120

FIGURE 8.—Section of lactating cells from normal mammary gland of tumor-resistant strain C57BL mouse. Lumen at *right* contains many dense particles assumed to represent solids of milk. They appear to be derived from within cytoplasm where they form in vacuoles and are liberated by a reversed pinocytosis. These particles liberated from lactating cells vary greatly in size and have no internal morphology as do the tumor milk-agent particles. × 15,000

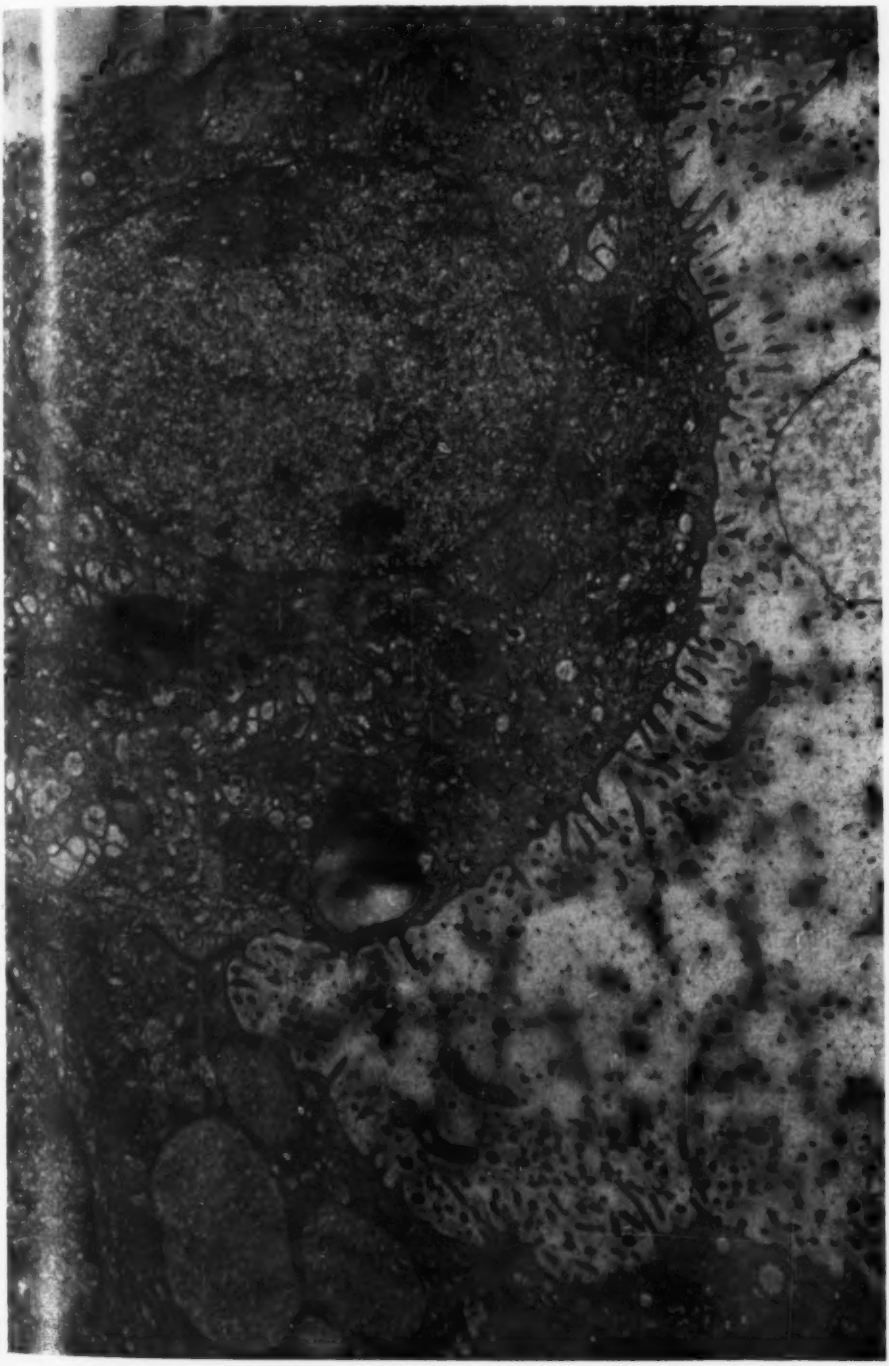
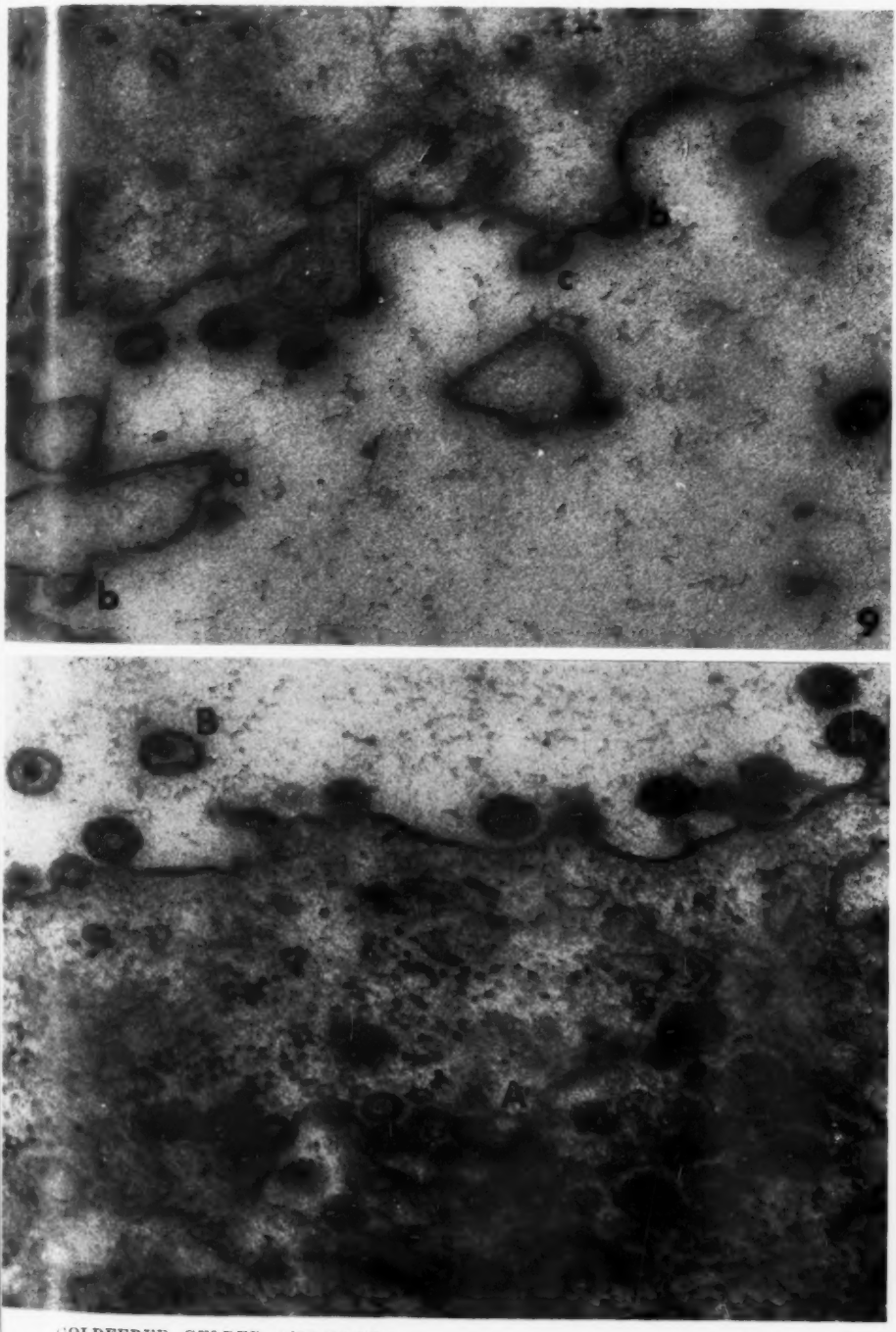


PLATE 121

FIGURE 9.—Electron micrograph showing detail of partially formed type B particles. Initial stages of formation are believed to be represented by *a*, and later stages by *b* and *c*. Material of new particle seems here to accumulate entirely within the cell membrane and half-formed inner dense shell of particle is clearly seen at *a*: at *b* dense shell is complete but semidense material between outer membrane and inner dense shell is not complete on proximal side of particle. This structure can give false appearance of a type A particle being enveloped in plasma membrane as it is liberated. × 77,000

FIGURE 10.—Micrograph showing detail of type A and type B particles. A particles seem to be forming from a semidense matrix deep within cytoplasm, while B particles are forming at cell membrane. Larger bodies at *lower right* appear to be of type B, immature, but may be atypical of either type. × 77,000


b
c
a
b
9
B
A

Development of Plasma-Cell Neoplasms In BALB/c Mice After Intraperitoneal Injection of Paraffin-Oil Adjuvant, Heat-Killed Staphylococcus Mixtures [1]

MICHAEL POTTER *and* CHARLOTTE L. ROBERTSON,[2] *Laboratory of Biology, Leukemia Studies Section, National Cancer Institute,*[3] *Bethesda, Maryland*

SUMMARY

Plasma-cell neoplasms developed in 7 of 64 mice inoculated intraperitoneally with an emulsion of incomplete Freund's adjuvant and heat-killed staphylococci. Thirty-two mice received only the injection; 4 plasma-cell neoplasms developed in this group. The remaining 32 mice received in addition to the injection a series of skin paintings with methylcholanthrene; 3 plasma-cell neoplasms developed in this group. All the other methylcholanthrene-painted mice developed carcinomas of the skin. Four of the 7 plasma-cell neoplasms were successfully transplanted. Mice with transplants developed serum myeloma globulins similar to those found in the original mouse. The role of the paraffin-oil staphylococcal emulsion in the production of the plasma-cell neoplasms was not definitely established. This point is briefly discussed.—J. Nat. Cancer Inst. 25: 847–861, 1960.

SPECIFIC MECHANISMS in the evolution of plasma-cell neoplasms in mice are not known. Until recently, these neoplasms have been rare. The observation that they developed in BALB/c mice, after the implantation of a Millipore diffusion chamber containing tissue from a C3H mouse, opened a new experimental approach to the problem of the genesis of plasma-cell neoplasia (*1*). The two most important features of this work were: (1) A new inbred strain had been introduced, and (2) the neoplastic process appeared to develop as a consequence of the interaction of host and complex foreign materials in the intraperitoneal cavity. Previously, plasma-cell neoplasms arising in the ileocecal area had been found to develop in C3H/He mice, and related substrains, during their domestic life in laboratory cages (*2*, *3*). No method of predicting or directing their occurrence has been devised. Rask-Nielsen reported plasma-cell leukemia in various inbred and hybrid mice, the incidence of which was increased by the administration of carcinogenic hydrocarbons (*4*).

An attempt was made to find another means of producing plasma-cell

[1] Received for publication May 3, 1960.

[2] The authors wish to thank Miss Rose Lieberman for the preparation of the adjuvant, heat-killed staphylococcus mixtures.

[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

neoplasms in BALB/c mice. The plan adopted was (1) to induce a proliferation of plasma cells by introducing a bacterial suspension in an adjuvant emulsion [antigen (*5*)] and (2) to superimpose a carcinogen. The carcinogen selected was methylcholanthrene, to be applied to the skin by a series of paintings. The antigen-adjuvant emulsion, described by Lieberman *et al.* (*6*), consisted of a mixture of incomplete Freund's adjuvant and heat-killed staphylococci. They reported that this adjuvant consistently produced in mice ascitic fluid, containing antibody (*7*). Plasma-cell neoplasms were found not only in the methylcholanthrene-painted mice but also in the mice that received only the adjuvant, heat-killed staphylococcus injection.

A brief report on the first 7 neoplasms is the subject of this paper.

MATERIALS AND METHODS

The mice used were strain BALB/cAn, bred by the Animal Breeding Unit of the Laboratory Aids Division, National Institutes of Health. They were placed in plastic cages, 8 mice per cage, and fed Derwood pellets and tap water *ad libitum*. Males and females were used for transplantation purposes, but only female mice were used in the long-term experiment. 3-Methylcholanthrene (MCA, a 0.2% solution dissolved in diethyl ether) was applied with a #6 camel's-hair brush, 3 times weekly, until 20 paintings had been given. The painting site was rotated through 12 different regions. The details of the technique of subcutaneous tumor transplantation were previously described (*8*).

The paraffin-oil staphylococcus emulsion was made as follows: Bayol F,[4] 8.5 parts, and Arlacel A,[5] 1.5 parts, were mixed in a Waring blendor until no separation occurred. This mixture, known as incomplete Freund's adjuvant, was autoclaved for 15 minutes at 121° C., tested for sterility, and stored at 4° C. A trypticase soy-broth culture[6] of *Staphylococcus aureus*, strain 18 (*6, 7*), was prepared as follows: One ml. of an 18-hour culture of *S. aureus* in trypticase soy was inoculated into a flask containing 3000 ml. of trypticase soy broth. This culture was incubated at 37° C. under continuous aeration for 18 hours. It was transferred to a 60° C. water bath for 1 hour, then stored at 4° C., and on the following day placed a second time in the 60° C. water bath. The culture was tested for sterility, adjusted to contain 16×10^9 bacterial cells per ml. with turbidometric and centrifugal methods, and stored at 4° C. Just prior to inoculation of mice, equal parts of the incomplete Freund's adjuvant and heat-killed trypticase soy-broth culture of *S. aureus* were emulsified. A piston-type arrangement with two connected syringes was used. The two components were mixed until no separation occurred.

[4] Bayol F was made by Esso Standard Oil Co. of New Jersey.
[5] Arlacel A (mannide mono-oleate) was made by Atlas Powder Co., Wilmington, Del.
[6] Trypticase soy-culture medium was purchased from Baltimore Biological Laboratories, Baltimore, Md.

A single injection of 0.3 ml. was given intraperitoneally to each of the 64 mice. This amount was chosen because it was less than the amount required to produce ascites but still adequate for inducing antibody (*6*).

Electrophoresis was carried out in a Spinco paper-electrophoresis apparatus, in 0.05 M Veronal buffer *p*H 8.6, for 16 hours at room temperature. Tracings of the strips were made on an Analytrol (*8*). Ten λ of serum was applied to each strip.

Tissues for histology were fixed in Tellyesniczky's fluid or Zenker-formol solution and the sections stained with hematoxylin and eosin.

When they were 6 weeks of age, 64 BALB/cAn female mice received intraperitoneal injections of 0.3 ml. of the adjuvant, heat-killed staphylococcus emulsion. Thirty-two (group I) were given a series of paintings with methylcholanthrene, beginning 1 week after the injection of the adjuvant, staphylococcus emulsion; the other mice received no further treatment (group II).

RESULTS

Seven of the 64 mice developed plasma-cell neoplasms, 3 in group I and 4 in group II. Pertinent information is given in table 1. All the plasma-cell neoplasms in group II occurred in cage mates; none developed in mice in the 3 other cages. Since the mice were assigned to cages randomly at the onset of the experiment, litter mates may or may not have been separated.

TABLE 1.—Data on origin and transplantation of plasma-cell neoplasms

Neoplasm	Origin			Transplantation			Electrophoretic mobility of myeloma globulin
				Number positive/number inoculated			
	Age of mouse (*months*)	Group	Cage	Generation 1	Generation 2	Generation 3	
Adj-PC-1*	7½	MCA†	6	0/4	—	—	
Adj-PC-2	7½	—	3	5/5	4/33	2/12	β (fast) diffuse
Adj-PC-3	9½	MCA	9	0/5	—	—	
Adj-PC-4	10	—	3	0/7	—	—	
Adj-PC-5	10½	—	3	2/2	15/16	10/10	γ narrow
Adj-PC-6	10½	—	3	4/7	19/21	13/13	β (slow) diffuse
Adj-PC-7	11	MCA	8	3/14	9/10	3/4	β

*Adjuvant-plasma cell.
†3-Methylcholanthrene.

Twenty-five of the 32 original mice in group II were alive at 15 months of age. Other causes of death were acute effects of the adjuvant injection, intestinal obstruction (at 14 months), and lymphocytic neoplasm (at 15 months).

Four of the 7 plasma-cell neoplasms were successfully transplanted subcutaneously (table 1). The other 3 have failed to grow on transplanta-

tion, though the recipient mice were observed more than 4 months. The transplantable plasma-cell neoplasm Adj-PC-2 grew progressively in all 5 hosts of the first generation but failed to grow in all hosts of the second and third transfer generations. The 5 first-transfer generation lines were each transplanted separately. None of the 5 lines have grown progressively in all recipients (table 1).

One of the first-transplant generation lines of the plasma-cell neoplasm Adj-PC-2 was transplanted to 14 female mice. Seven of these had been conditioned by an intraperitoneal injection of 0.5 ml. of the adjuvant, staphylococcus emulsion 1 month previously. The other 7 had no previous treatment. The only successful transplant occurred in one of the conditioned mice. The original Adj-PC-7 neoplasm was transplanted to 8 untreated mice and 6 conditioned mice (4 conditioned mice had received intraperitoneal injections of 0.5 ml. adjuvant, staphylococcus emulsion 6 weeks previously, and 2 had received intraperitoneal injections 10 days previously). Progressive growth of the subcutaneous transplant occurred in only 3 of the untreated mice.

The electrophoretic mobilities of the myeloma globulins associated with the transplantable plasma-cell neoplasms are given in table 1 and text-figure 1. Three of the peaks of globulin concentration were found in the β-region, one in the γ (Adj-PC-5). The γ-mobility globulin was concentrated in a narrow portion of the paper strip; the β-mobility globulins were more widely spread. Adj-PC-2 was associated with a broad band of increased globulin concentration in the β- and fast β-region, while Adj-PC-6 was associated with increased concentration of globulins in the γ and slow β-mobility region.

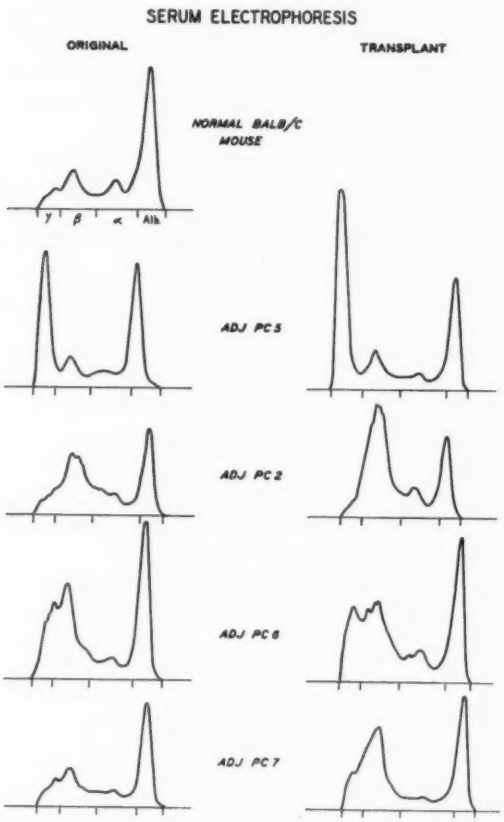
The difference among the mobilities of the myeloma globulins of Adj-PC-2, -5, and -6 was marked. These mice received a similar treatment (group II) and were housed in the same cage.

In text-figure 1, the electrophoretic patterns of the original serum and serum from a mouse bearing a transplant are given. In each of 3 pairs of serums, the areas of increased globulin concentration in original and transplant are similar. In the fourth example, Adj-PC-7, no peak of excessive globulin was found in the original host, while the serum of the mouse bearing a transplant had a large peak of globulin concentration in the β-region.

DESCRIPTION OF PRIMARY PLASMA-CELL NEOPLASMS

Macroscopic.—All mice with plasma-cell neoplasms developed ascites. Five had 4 to 8 ml. of bloody ascites, the other two, 1 ml. or less. In all mice, the ascitic fluid contained cells of a characteristic type (*see* description to follow) which permitted antemortem diagnosis.

The peritoneal surface of the mesentery was studded with many nodules of varying size (1 to 20 mm. in diameter, fig. 1). The nodules were irregularly scattered throughout the mesentery and were located fre-



TEXT-FIGURE 1.—Electrophoretic patterns of original serum and serum from mouse bearing a transplant.

quently near the gut. Some mice had small nodules (5 mm. or less in diameter) but others had large nodules (1 to 2 cm. in diameter) in addition to the small ones. Two mice had a mass of retroperitoneal tissue containing neoplastic tissue, which suggested that the injection of the adjuvant had been deposited there.

Microscopic.—The neoplastic plasma cells of the ascites resembled the tumor cells found in the ascites forms of the transplantable plasma-cell neoplasms described previously, MPC-1, -2, -3, and -4 (*8*).

The characteristic ascites tumor cell was large and at least 6 to 8 times the size of the small lymphocytes and granulocytes seen in the same fields. The cell outline was irregular, neither round nor oval. Although the cytoplasm was deep blue, a fine reticulation was discernible. A juxtanuclear translucent region, varying in size and shape, was seen in the cytoplasm of most cells. The nucleus was almost always either lobulated or duplicated. Small bridges of nuclear material connected the

lobes. Mitotic figures were found in cells having the size and staining characteristics of neoplastic plasma cells. Some of the ascitic fluid contained numerous cells of this type, but in others only a few scattered cells were found. All ascitic fluid contained histiocytes, lymphocytes, and granulocytes besides plasma cells.

Two pathologic processes were found in the tissues of mice which developed plasma-cell neoplasia: (1) a lipogranulomatous reaction and (2) a proliferation of neoplastic plasma cells.

The lipogranulomatous tissue in all mice which received the adjuvant, staphylococcus emulsion adhered to peritoneal and visceral surfaces and consisted of numerous cystlike spaces and a reactive connective tissue (fig. 2). The cysts were of variable size; some had thinly lined walls while others had thickened walls composed of endothelial-type cells, histiocytes, and large cells with a "foamy" cytoplasm. Often granulocytes were scattered in the lining. The cysts appeared to be cavities which had contained paraffin oil extracted during the clearing process.

The interstitial tissue in some places consisted of eosinophilic fibrinoid material containing only a few cells. In most areas it was populated by clusters of cells (lymphocytes), rarely, plasma cells, or cells scattered randomly throughout (lymphocytes, granulocytes, and histiocytes). In the mice with plasma-cell neoplasms, the lipogranulomatous tissue was largely replaced by neoplastic plasma cells. Many nodules were seen, probably due to seeding of the peritoneal surface by cells that had detached from other regions.

Areas suggesting possible morphologic transformation from granulomatous tissue to a plasma-cell neoplasm were observed in 2 of the mice (one of these areas is shown in fig. 3). The interstitial tissue between vacuolar cysts contained plasma cells. The small colonies, which contained several hundreds of these cells in one section, were completely surrounded by lipogranulomatous tissue. However, serial sections would be necessary to prove whether these colonies of plasma cells may have been independent or whether they were attached to a mass of plasma cells on the surface of the peritoneum.

The neoplastic plasma cells had a variety of cytologic appearances (figs. 4, 5, and 6). In most cells the cytoplasm was densely stained and contained a clear juxtanuclear zone that was chromophobic. The nucleus was often lobulated or duplicated and contained densely clumped chromatin. Some cells had pyknotic nuclei.

The lymph nodes of 6 mice with plasma-cell neoplasms were invaded. The usual pattern consisted of numerous plasma cells in the marginal and hilar sinuses of the gland (fig. 7). The medullary cords were involved extensively in one node in one mouse, but in the others the infiltration was negligible or slight. Uninvolved nodes were frequently found in the adjacent tissue or in the same mouse. The spleen and liver did not contain neoplastic cells.

The walls of the cecum were not infiltrated with neoplastic plasma cells, *i.e.*, macroscopically or microscopically. The cecum in 5 of the 7 cases,

however, contained protozoa and helminths in the lumen. The parenchyma of the abdominal viscera, kidneys, and lungs were not infiltrated with plasma cells. No evidence of "myeloma kidney" was found.

The mouse in which the plasma-cell neoplasm Adj-PC–7 developed had, in addition, several papillomas and carcinomas of the skin, a pulmonary adenoma, and a histiocytoma of the lymph node. The neoplastic tissue of the first-generation transplant, however, was composed of plasma cells.

Papain-digested skeletons were prepared from all the original mice except Adj-PC-2. No osteolytic lesions were found. Four mice bearing transplants of Adj-PC-2 had normal skeletons.

CONCLUSION

It is not possible to implicate directly the paraffin-oil staphylococcus emulsion, as the inducer of the 7 plasma-cell neoplasms, because of the few cases and the lack of controls.

Four of the 32 mice that received only the adjuvant, staphylococcus emulsion developed plasma-cell neoplasms. The meaning of this can only be interpreted when a suitable control procedure is devised.

The major finding that appeared to implicate the adjuvant, staphylococcus emulsion as having played an important role in the genesis of the plasma-cell neoplasms was the extensive involvement of the lipogranulomatous tissue with plasma-cell neoplasm. In certain mice, evidence of a transition between lipogranulomatous tissue and plasma-cell neoplasm was observed.

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PLATE 122

FIGURE 1.—Mouse with original plasma-cell neoplasm (Adj-PC-3). Mesentery greatly thickened by numerous ill-defined nodules.

560070—60——18

PLATE 123

FIGURE 2.—Peritoneal surface of gut from mouse that did not develop plasma-cell neoplasm. Lipogranulomatous tissue with numerous cystlike spaces is firmly attached to serosal surface of intestinal wall. × 135

FIGURE 3.—Lipogranulomatous tissue from mouse with plasma-cell neoplasm (Adj-PC-5) with collection of neoplastic plasma cells. *Note* comparative size of small lymphocytes. × 200

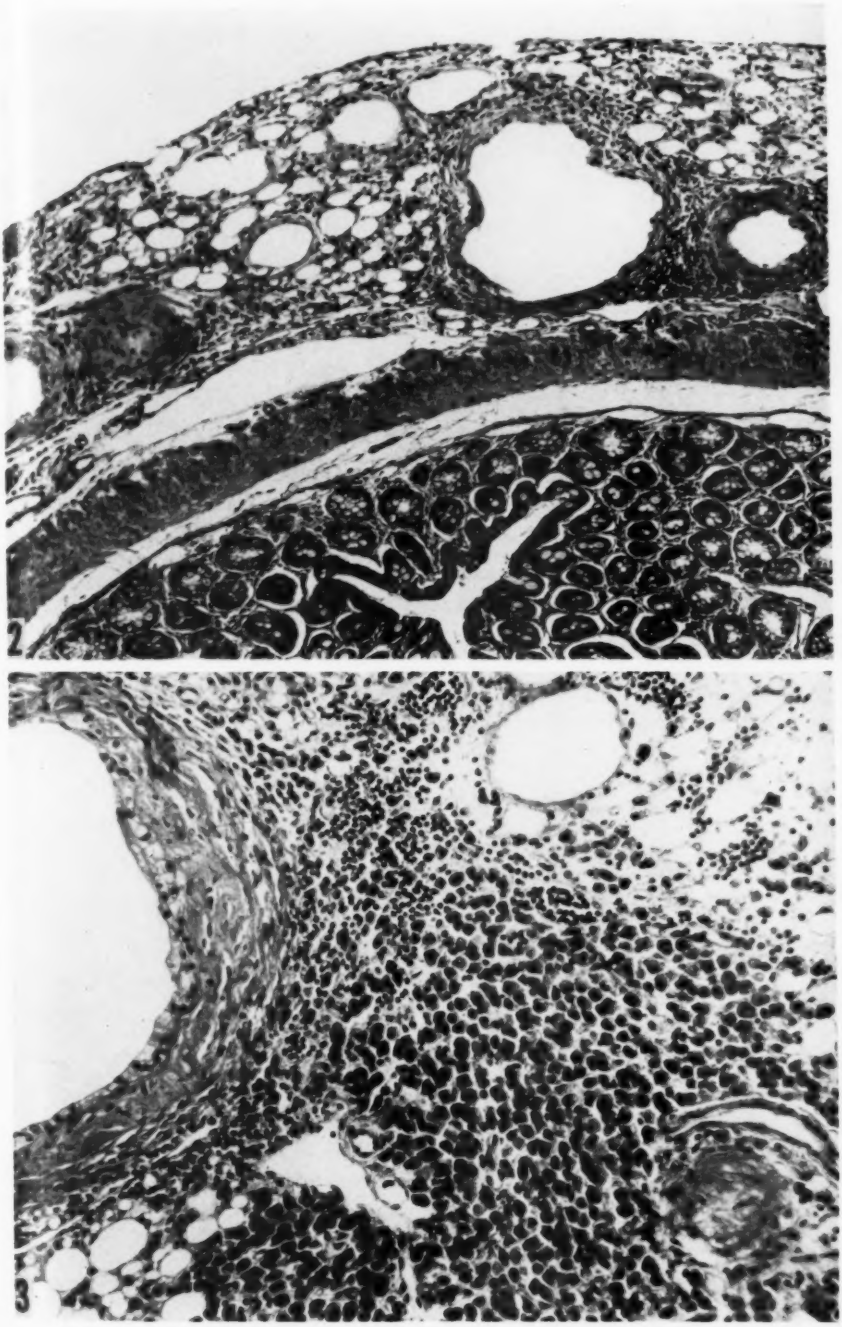


PLATE 124

FIGURE 4.—Lipogranulomatous tissue infiltrated with neoplastic plasma cells, original plasma-cell neoplasm Adj-PC-2. × 830

FIGURE 5.—Infiltration of lipogranulomatous tissue with neoplastic plasma cells *Note* relative size of small lymphocytes (Adj-PC-5). × 510

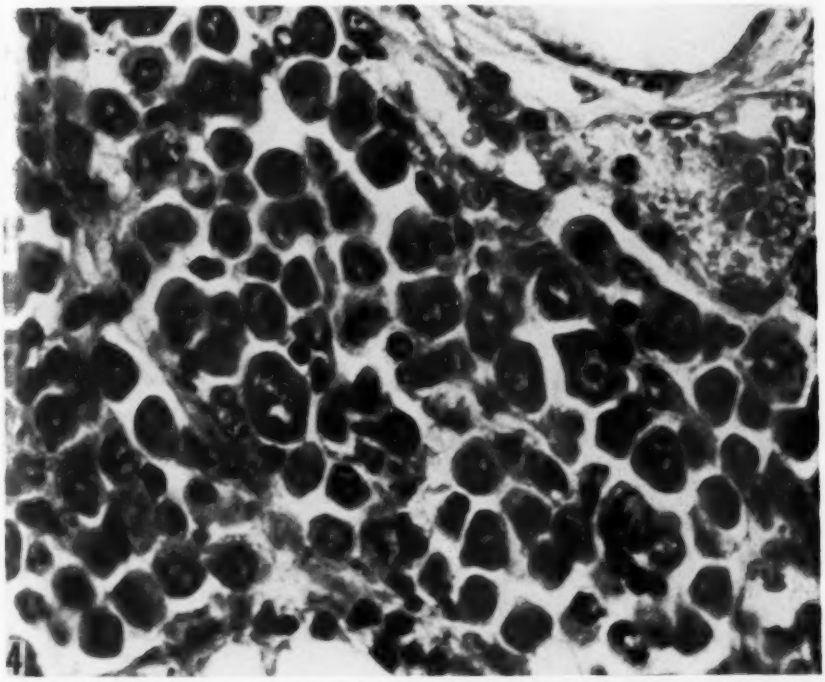
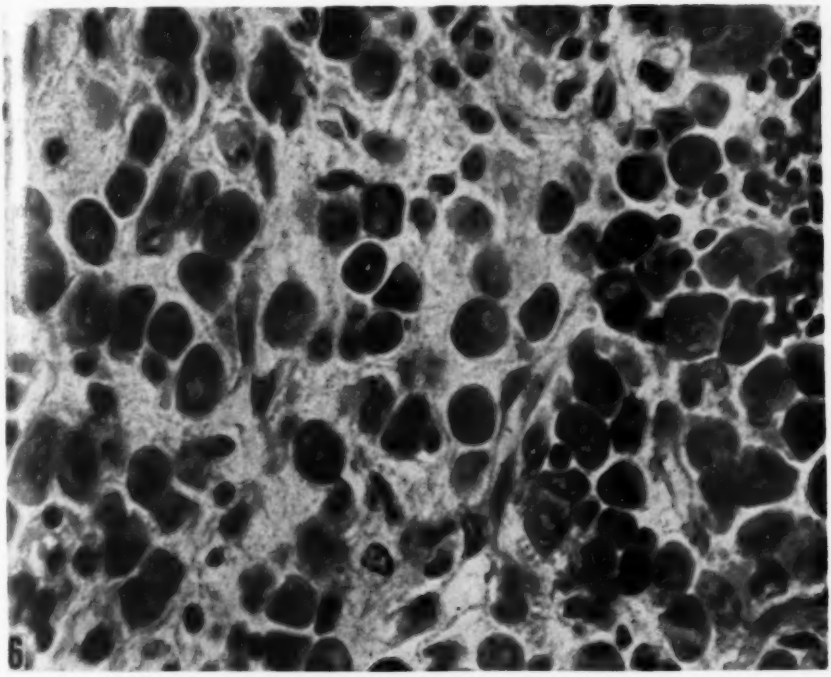
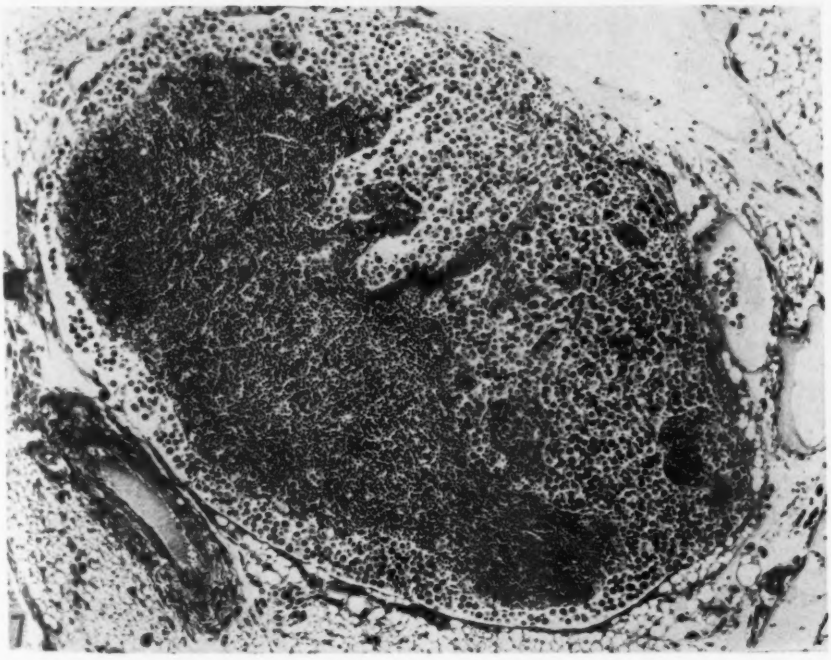


PLATE 125

FIGURE 6.—Collection of neoplastic plasma cells. Juxtanuclear clear zone is seen clearly in 3 cells in horizontal midline of field. *Note* variable amount of pyknosis in nuclei. Adj-PC-5. × 780

FIGURE 7.—Lymph node from mouse with original plasma-cell neoplasm Adj-PC-6. Numerous large neoplastic plasma cells appear in marginal and hilar sinuses. × 95





Initial Effect of Community-Wide Cytologic Screening on Clinical Stage of Cervical Cancer Detected in an Entire Community. Results of Memphis-Shelby County, Tennessee, Study [1, 2]

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SUMMARY

Cytologic technique was applied to vaginal aspiration smears taken from 151,000 women, including 67.6 percent of the white and 57.3 percent of the Negro population of Memphis and Shelby County, Tennessee, 20 years of age and older. At first screening performed during the 5 years beginning July, 1952, 1.3 cases of unsuspected invasive cervical cancer were detected in each 1,000 white residents screened, and 1.8 cases per 1,000 Negro residents. The prevalence ratios for unsuspected intra-epithelial carcinoma (carcinoma *in situ*) detected were 3.1 per 1,000 white residents and 4.0 per 1,000 Negroes. The prevalence of unsuspected invasive disease, taken in connection with the presurvey yearly incidence of newly diagnosed cases, provided an estimate that the cytologically detected cases were probably identified, on the average, about 3 years earlier in their invasive phase than the cases diagnosed in the presurvey period. Benefit of the program to the community is indicated by diagnosis of invasive disease at an earlier stage in the entire population. Prior to the screening program, 34 percent of all white patients and 18 percent of all Negro patients in Memphis and Shelby County were in clinical stage I at diagnosis. During the program these proportions increased to 57 and 38 percent, respectively, for the entire population, and to 86 and 77 percent, respectively, for unsuspected cancer cases detected by the screening technique.—J. Nat. Cancer Inst. 25: 863–881, 1960.

BETWEEN JULY 1, 1952, and June 30, 1957, vaginal cytology examinations were offered for the early detection of cancer of the uterine cervix among the women of Memphis and Shelby County, Tennessee. During this 5-year period Papanicolaou (*1*) smears were taken by vaginal aspiration technique on one or more occasions from 151,000 women. It is the

[1] Received for publication May 20, 1960.

[2] This study was supported in part by grants CS-9210, 9211, 9213, 9280, and 9439 from the Field Investigations and Demonstrations Branch. It could not have been accomplished without the unstinting cooperation of numerous individuals and organizations. In particular, the cooperation of the many individual physicians, the Memphis and Shelby County Medical Society, and the area pathologists is gratefully acknowledged.

[3] Field Investigations and Demonstrations Branch, National Cancer Institute, National Institutes of Health, Public Health Service.

[4] Division of Pathology and Microbiology, University of Tennessee, Memphis, Tenn.

[5] Division of Obstetrics and Gynecology, University of Tennessee, Memphis, Tenn.

[6] Memphis-Shelby Co. Health Dept., Memphis, Tenn.

purpose of this paper (1) to review briefly the objectives of this first large-scale community-wide application of female genital cytologic examination; (2) to present final data, preliminary summaries of which have been reported previously (*2–6*); (3) to call attention to some of the problems encountered in community-wide screening as they relate to data presented; and (4) to note the immediate beneficial effect on the clinical stage of cervical cancer diagnosed in this defined community. Subsequent communications will deal in more detail with prevalence and incidence of uterine cancer in various segments of the population, both screened and nonscreened, and with inferences regarding some aspects of the epidemiology and pathogenesis of the disease which may be drawn from the results.

OBJECTIVES OF STUDY

From its inception in July, 1947, one of the objectives of the Field Investigations and Demonstrations Branch of the National Cancer Institute (orginally called the Cancer Control Branch) has been the stimulation, development, and testing of methods proposed for the early detection of cancer. At the time the Branch was organized the Papanicolaou test, as applied in office and clinic practice of medicine, had already demonstrated its usefulness as an adjunct in the diagnosis of cervical cancer. Consideration was given to possible extension of application of the technique by studying feasibility of its use as a screening device in large population groups. Discussions with pathologists at the University of Tennessee in Memphis, where a similar interest had developed, led, in 1951, to establishing in Memphis and Shelby County, Tennessee, a joint cooperative study with the University of Tennessee for a community-wide use of cytologic examination as a screening device.

Applicability of a mass screening test depends on a number of factors besides feasibility and practicality. Not the least of these is the cost to the community in terms of "false alarm" and "false sense of security" (*7*) which mass application might engender. The study was therefore designed to collect more specific information about the sensitivity and specificity of the test on a mass basis. By 1951 it had also become clear that different investigative methods, additional to those then in use, were necessary for a clearer understanding of the quantitative relationship between intra-epithelial carcinoma (carcinoma *in situ*) and invasive cancer of the cervix. The epidemiological model designed by Dunn (*8*) describes this approach and study plan. This involved considerations of the interplay of prevalence and incidence of the two lesions in large samples of the general female population observed over a period of time, prevalence being the proportion of women with a lesion at initial examination, and incidence being the proportion with new lesions at a subsequent examination. Successful testing of the epidemiological model requires stable incidence rates, which in turn requires repeated

rescreening of large numbers of women who did not have lesions at first screening.

The broad objectives of the study included both operational and fundamental research. From the standpoint of operations, the chief questions raised involved: (1) the feasibility of mass screening with the cytologic examination; (2) assay of its value in early detection of cancer and intra-epithelial carcinoma in an entire community; (3) practical problems encountered; and (4) cost to the community in terms of false alarm and false sense of security. From the standpoint of basic research, observations were made with the hope of clarifying some aspects of the pathogenesis and epidemiology of this disease.

The details of organization and conduct of the study have been described (*9*). After a year of preparation and a general publicity campaign directed by the University of Tennessee, the research project was started in July, 1952.

NUMBER AND COMPOSITION OF POPULATION EXAMINED

During the 5-year-period beginning in July, 1952, a total of 290,000 smears were taken for 252,000 examinations of 150,951 women. The initial smear was classified as unsatisfactory for accurate evaluation in 14.9 percent of the first 52,000 examinations (1952–53). The percentage decreased, as the study progressed, to 8.1 percent of the 200,000 examinations done in 1954–57. Thin smears (too few cells for accurate evaluation) accounted for more than two thirds of unsatisfactory smears, and cellular spreads considered too thick or containing too many blood cells or inflammatory cells were responsible for the balance of examinations classified as unsatisfactory for accurate cytologic evaluation. While a single vaginal specimen was requested for the routine screening cytology examination, for all women with tests considered unsatisfactory on first testing, both vaginal and cervical (aspiration and scraper) specimens were requested on the repeat examination (*2–6*). Of the women recalled for repeat smears, 6,229 did not return. Thus, 4.1 percent of the total number of women did not have satisfactory observation, but 144,722 women with one or more smears were considered satisfactory for accurate cytologic assessment.

Table 1 shows the women participating in the study, by residence, race, and year of first examination. Nonresidents of Memphis and Shelby County comprise 15.6 percent of the participants. Most were drawn from adjoining counties of Tennessee, Mississippi, and Arkansas; many were women who depend on Memphis physicians for their medical care; others were patients referred because of gynecological problems, including referrals to the West Tennessee Cancer Clinic, a regional facility. Their participation could not be refused in a community-wide screening project when public emphasis was on case finding, and they must be considered in any analyses dealing with operations research. They cannot be in-

cluded when considering the effect of mass screening on uterine cancer in the Memphis-Shelby County population.

TABLE 1.—Number of women participating in first screening, by residence, race, and year of first examination*

Year	Memphis-Shelby County residents			Nonresidents			Grand total
	White	Negro	Total	White	Negro	Total	
1952†	5, 699	8, 181	13, 880	800	185	985	14, 865
1953	21, 460	10, 906	32, 366	3, 333	383	3, 716	36, 082
1954	16, 600	7, 350	23, 950	4, 105	404	4, 509	28, 459
1955	17, 001	5, 622	22, 623	4, 927	364	5, 291	27, 914
1956	17, 640	6, 018	23, 658	5, 592	366	5, 958	29, 616
1957†	8, 343	2, 643	10, 986	2, 871	158	3, 029	14, 015
Total	86, 743	40, 720	127, 463	21, 628	1, 860	24, 388	150, 951

*Including 551 women with uterine or other pelvic cancer diagnosed prior to cytologic examination; 6,229 for whom the only observation was a single unsatisfactory cytologic smear; and 541 whose cancer was suspected at time of screening (total = 7,321). The number at risk of discovery of unsuspected cancer is thus 143,630 (150,951 − 7,321).

†For 6 months only: July to December, 1952, and January to June, 1957.

Among the 127,463 resident participants (table 1) 86,743 were white and 40,720 were Negro. The necessity for treating each race separately reduces the effective number of screenees for some analyses. Having two groups in which the disease apparently behaves somewhat differently does offer certain advantages which will be evident later.

Table 1 also shows that the largest number of women appeared for first screening during 1953. This was due partly to the intensive publicity campaign conducted during the early phases of the study, and partly, of course, to the fact that as the project progressed there were fewer unscreened women available for first screening. It seemed that the number of examinees appearing was largely a direct response to the tempo of general publicity. Accordingly the attempt was made to increase or decrease publicity to balance the volume of examinations received in relation to facilities available for examination and reporting and especially to the current number of cytotechnicians available. This was necessary to facilitate prompt reporting of cytology examinations to the cooperating physicians. Every effort was made to notify physicians of cytology results as rapidly as possible after receipt of the smear in the laboratory, with specific recommendations for further study of those with abnormal cells.

The number of smears processed in the laboratory each week averaged 1,250 and ranged from a minimum of 300 smears, during a typical low week (Christmas holiday, December, 1952), to a maximum of 1,933 (January, 1957). The processing was necessary for smears taken at initial examination of 152,000 women, for repeat smears found unsatisfactory and abnormal (which included cervical scrapings and cervical aspirations as well as vaginal specimens on repeats), and for rescreening

those who returned "annually." The cytotechnologists available to handle this volume of smears, including the checkers and senior technologist, ranged from 9 to a maximum of 18. In relating the number of cytotechnologists to the number of smears examined, extreme fluctuation in workload must be considered as well as a turnover in laboratory personnel, which was so great that it was necessary for the University to provide on-the-job training and to operate a training school for cytotechnologists on a continuing basis.

Table 2 shows the number of white and Negro women 15 years of age and older estimated as residing in Memphis and Shelby County at the midpoint of the study period, and the number and percentage in each group with one or more satisfactory observations during the 5-year period. Although the general publicity for case finding was directed toward women 20 years of age and older, 7,163 (5.8 percent of the total) were under 20. Among residents 20 years of age and older, 67.6 percent of the white and 57.3 percent of the Negro had one or more satisfactory examinations. The proportion examined decreased rapidly with age, so that only 21.6 percent of the white and 17.2 percent of the Negro residents 65 years old and older participated. Thus, the response to the case-finding program was greatest among those in the age groups least at risk to invasive disease and poorest among those in the ages of greatest risk. A similar type of participation by age has been observed in mass chest X-ray surveys. Any effort in the future for exploiting vaginal cytology as a device for detection of invasive disease must include some means to increase the participation of women in the ages of greatest risk to invasive cancer of the cervix.

TABLE 2.—Estimated resident female population of Memphis and Shelby County, Tennessee, by race and age, and number and percent with one or more satisfactory cytologic examinations during 5 years, July, 1952 to July, 1957

Age	Number of resident women*		Number of women examined		Percent	
	White	Negro	White	Negro	White	Negro
15–19	11,518	7,638	4,022	3,141	34.9	41.1
20–34	44,958	22,276	40,262	19,751	89.5	88.6
35–49	35,759	20,501	25,704	10,757	71.8	52.4
50–64	22,694	12,748	10,407	4,288	45.8	33.6
65 and over	13,993	7,467	3,019	1,281	21.6	17.2
Totals:						
15 and over	128,922	70,630	83,414	39,218	64.7	55.5
20 and over	117,404	62,992	79,392	36,077	67.6	57.3

*Estimated for January 1, 1955, the midpoint of the study period, by interpolation between the 1950 census and the special census conducted by the Bureau of the Census in Memphis and Shelby County on January 31, 1958.

The study, as planned, envisioned cytologic screening, with diagnostic follow-up on those with abnormal cytologic findings, of as many women 20 years old and over as could be attracted to participate from the entire population of Memphis and Shelby County. It was recognized that

coverage of the entire population was a Utopian dream not likely to be realized. Some major objectives of the study, however, required large numbers of examinees who were representative of the population as a whole. In addition, it was necessary that some of the well-known selective factors (*10*) bearing on the distribution of cancer of the cervix be accounted for to permit valid inferences to be drawn from the data derived. One indirect indicator of some of these factors is the place where the smear was collected.

Women who are accustomed to seeking regular medical care and supervision from private physicians represent a different cervical-cancer risk than the medically indigent population cared for at the Gailor-Gaston (City of Memphis) hospitals and clinics, or by other medical agencies. Both groups, however, could be reached through existing channels by a screening program. The group actually screened at Gailor-Gaston included many who came for "tests," the food-handler group, and visitors as well as patients in clinics and wards. A high proportion of women attending antepartum clinics also had cytologic observations. The follow-up and clinical management of medically indigent women with suspicious cytology findings were the responsibility of the staff at the University Division of Obstetrics and Gynecology.

Other medical sources consisted of a few small outpatient facilities plus State and county institutions, and included the West Tennessee Cancer Clinic, a referral center for the region, where vaginal smears were routine for all women seen for any cause.

Special facilities were provided for collection of specimens at locations and times convenient to groups of interested women who belonged to neither the private patient nor the medically indigent group and who might be reluctant to go to a physician's office for a test. These facilities included special clinics set up in cooperation with the health department in various parts of the city and county, usually at schools, and industrial clinics at a number of commercial and industrial installations where collection of specimens was satisfactorily undertaken by clinic aides trained and supervised by a Public Health Service nurse under the direction and supervision of gynecologists of the University of Tennessee. In the special clinics, each participant was asked for the name of her family physician, to whom all suspicious or questionable reports were sent. The patient was notified through her physician if the test result was not negative.

Table 3 shows the number of women by source of referral at first examination, by residence and race. Private physicians collected the first specimens from 61 percent of the white residents, and from more than one half (52%) of the total 143,630 participants who might have been harboring an unsuspected cancer. The largest number of Negro residents (74%) were seen at Gailor-Gaston. Only 1.6 percent of the smears from residents came from other medical sources. The remainder of the residents, 30 percent of the white and 9 percent of the Negro participants, were first seen at one of the special clinics, designated in

the table as special and industrial. Total numbers at these clinics were not as large as could have been desired, and the population in some was relatively unstable because of the inclusion of many participants from Millington Naval Air Station, Mallory Air Force Depot, and other smaller groups subject to high transfer rates.

TABLE 3.—Source of referral, at first screening, of women potentially at risk of detection of unsuspected cancer*

Source of referral	Memphis-Shelby County residents			Nonresidents			Grand total
	White	Negro	Total	White	Negro	Total	
Private physician	50, 507	5, 970	56, 477	17, 779	500	18, 279	74, 756
Gailor-Gaston (City of Memphis hospitals)	6, 202	28, 865	35, 067	524	472	996	36, 063
Other medical agencies	1, 460	546	2, 006	910	546	1, 456	3, 462
Industrial clinics	10, 464	967	11, 431	207	10	217	11, 648
Other special clinics	14, 464	2, 564	17, 028	626	47	673	17, 701
Total	83, 097	38, 912	122, 009	20, 046	1, 575	21, 621	143, 630*

*All 150,951 women examined are potentially at risk of detection of unsuspected cancer, except the 551 already having a cancer diagnosis, the 541 whose cancer was suspected at the time of examination, and the 6,229 who had no satisfactory smear.

PROPORTION OF WOMEN HAVING RESCREENING EXAMINATIONS

At the outset of the study it was planned to recall for rescreening at annual intervals all women not found to have cervical cancer at their previous examination. The necessity for this was dictated by the requirements of the study plan (*8*) for stable incidence rates of invasive and intra-epithelial lesions of the cervix among women whose cytologic examination was negative at a prior screening. Accordingly, the files were periodically checked for those women who were due for rescreening. Sending recall notices was a clerical job of considerable size, complicated by current use of records and by the necessity of separating the records of examinees referred by private physicians, so that individual physicians could themselves sign and mail the notices prepared in the laboratory for their respective patients. Recall notices for other examinees were forwarded over the signature of the clinic physician or the senior pathologist. The tendency of many patients to have repeat smears taken by different physicians or at different clinics also contributed to the difficulty of assembling the recall notices.

At the beginning of the study, recall was delayed until more than a year had elapsed. Subsequent to this, though every effort was made to assemble and mail notices at annual intervals, the women did not always return for examination at such intervals. Many private patients returned regularly at 6-month intervals without recall notices; some had additional

smears at odd times because of a visit to a physician for another cause; many returned at intervals much longer than a year; and many more had only 1 smear. The irregularity of the time of the "annual" rescreenings creates no great difficulties in data analysis. The methods used and the results of the rescreening experience will be discussed in a subsequent paper. What is hereafter referred to as a rescreening, however, has been arbitrarily defined as a cytologic observation made 9 or more months after a previous smear.

With this definition of a rescreening in mind, it may be seen (table 4) that 42.6 percent of white residents had 2 screenings, 19.4 percent had 3, and 7.6 percent had 4. Percentages of Negro rescreenings were similar. Because of the time which must elapse between examinations to constitute a rescreening, it is clear that those first examined in the early portion of the study had a greater possibility of rescreening than those who entered the study later. Table 4 shows, for example, that 63.7 percent of the white women with first screening in the first 18 months (1952–53) were examined twice, while only 13.0 percent of those who first appeared in the last 18 months (1956–57) had a second screening. By definition, only those of the latter group who had their first screening in the first 9 months were eligible for rescreening. In addition, the total proportion rescreened was reduced because of a high rate of migration from the county.

TABLE 4.—Number and percent of resident women, with no positive diagnosis at first screening, who returned for a second, third, and fourth, by race and year of first screening

Year of first screening	Number without positive diagnosis at screen I	Number and percent rescreened					
		II		III		IV	
		Number	Percent	Number	Percent	Number	Percent
				White residents			
1952–53*	25, 874	16, 479	63. 7	10, 628	41. 1	5, 454	21. 1
1954–55	32, 017	15, 548	48. 6	5, 428	17. 0	864	2. 7
1956–57*	24, 835	3, 240	13. 0	2	0. 0	—	—
Entire period	82, 716	35, 267	42. 6	16, 058	19. 4	6, 318	7. 6
				Negro residents			
1952–53*	18, 168	11, 088	61. 0	6, 590	36. 3	2, 982	16. 4
1954–55	12, 274	5, 281	43. 0	1, 622	13. 2	209	1. 7
1956–57*	8, 235	928	11. 3	3	0. 0	—	—
Entire period	38, 677	17, 297	44. 7	8, 215	21. 2	3, 191	8. 3

*18-month period.

From the standpoint of stable incidence rates, which require a large number of rescreenings, the number of women who returned "annually" is somewhat disappointing. Since a mass screening of this kind has never been attempted before, it is not possible to say categorically whether the response in Memphis was "good" or "bad."

Total Cases Observed

Table 5 shows the number of cases of invasive cervical cancer, and table 6 the cases of intra-epithelial carcinoma of the cervix (carcinoma *in situ*), that were observed at first examination among all 150,951 women participating (table 1). It is noted that 658 patients with invasive cancer and 573 patients with intra-epithelial carcinoma of the cervix were seen. The cases listed in table 5 include 103 patients and those listed in table 6 include 16 patients known to have cervical carcinoma prior to cytologic examination in this study. "Detection" of previously diagnosed cases cannot, of course, be attributed to the screening program, nor can those cancers already suspected at the time of screening. Such cases are, however, part of the yield encountered in mass screening, and must therefore be considered in the logistics of program operations. Many represent legitimate follow-up for detection of recurrence of active disease in treated patients.

TABLE 5.—Number of cases of invasive cervical cancer observed at first screening of participants, by race, residence, and source of referral

Source of referral	Residents		Nonresidents		Total
	White	Negro	White	Negro	
Private physician	139	44	85	11	279
Gailor-Gaston (City of Memphis hospitals)	30	119	2	3	154
Other medical agencies	12	34	57	89	192
Industrial clinics	11	—	—	—	11
Other special clinics	20	2	—	—	22
Total	212	199	144	103	658*

*The total comprises (1) 103 previously diagnosed cases of active invasive disease, (2) 353 cases clinically suspected at the time of first cytologic examination, and (3) 202 cases which were unsuspected at the time of the initial cytologic observation.

It is common practice to take the cases observed in a cytologic examination experience such as this, and by using all cases seen (tables 5 and 6) as numerators, and the numbers participating (table 1) as denominators, to calculate ratios which are almost invariably designated as "incidence rates." Ratios computed in this way bear no relation to incidence, which is a measure of the rate of occurrence of an event. Such ratios do represent prevalence of a sort, but the only value which may be attached to this kind of prevalence ratio is what it might contribute to administrative considerations. Except in the administrative sense of the cases observed per 1,000 participants, ratios which might be computed cannot validly be compared to any other experience. A higher yield in one experience may reflect only a bias due to large numbers of women with symptoms, rather than a more efficient cytologic procedure or a greater actual prevalence of disease in the total population of which the examinees are only a part. For this reason, numbers of cases encoun-

tered are shown to indicate the volume of positive diagnoses, but prevalence ratios which might be derived are not presented.

TABLE 6.—Number of cases of intra-epithelial carcinoma of the cervix (carcinoma *in situ*) observed at first screening of participants, by residence, race, and source of referral

Source of referral	Residents		Nonresidents		Total
	White	Negro	White	Negro	
Private physician	174	26	73	3	276
Gailor-Gaston (City of Memphis hospitals)	32	136	5	4	177
Other medical agencies	6	2	9	5	22
Industrial clinics	39	4	1	—	44
Other special clinics	44	5	5	—	54
Total	295	173	93	12	573*

*The total comprises (1) 16 cases diagnosed prior to "first screening" cytologic examination, (2) 71 cases in which invasive or intra-epithelial carcinoma was suspected, and (3) 486 cases unsuspected at the time of cytologic examination. Of the total 573, 86 exhibited minimal histologic criteria for intra-epithelial carcinoma and were classified as "probable" intra-epithelial carcinoma.

DETECTION OF CLINICALLY UNSUSPECTED CANCER

From the standpoint of value of the test in early detection of cancer and of its usefulness in epidemiological research, the main interest centers around the unsuspected cancers discovered as a result of a suspicious cytologic observation. It was reasonable to expect that the publicity attendant upon the investigation would result in earlier clinical diagnosis, not only in the study group but among nonparticipants as well. Such indirect effects will be given consideration later in evaluation of the over-all usefulness of the program.

Each case of uterine cancer observed during the survey was classified as clinically suspected or unsuspected at the time of initial cytologic examination. Physicians were asked whether biopsy would have been done without knowledge of cytologic findings. Very few cases presented any problem in classification. These were investigated further before designation. It should be mentioned, perhaps, that "unsuspected" by no means necessarily implies "preclinical." The classification is based on the question, "Would the case have been diagnosed at this time?" and not, "Could it?" Some cases could certainly have been diagnosed clinically at or before the time of their detection by screening if the patients had sought medical attention.

Table 7 shows the prevalence of unsuspected invasive cervical cancer at first screening among residents 20 years of age and older, by race and age; age-adjusted ratios by residence, race, and source of referral are presented in table 8. The total prevalence among Negro participants was about 50 percent greater than among the white; the difference in

residents appears to be greater among older women than among those under 50 years of age. Further comment regarding the level of unsuspected prevalence and the clinical stage is included in the discussion of the total morbidity observed in the county.

TABLE 7.—Prevalence of unsuspected invasive cancer of cervix at first screening among Shelby County residents of age 20 and older, by race and age

Age	White			Negro		
	Number of cases	Ratio per 1,000	Number in stage I	Number of cases	Ratio per 1,000	Number in stage I
20–29	4	0. 15	4	6	0. 41	5
30–39	35	1. 44	33	20	2. 16	18
40–49	27	1. 80	26	17	2. 67	14
50–59	16	1. 98	11	16	4. 69	12
60–69	16	4. 24	10	6	4. 10	3
70 and over	6	4. 23	5	6	11. 34	2
Total	104	1. 32	89	71	1. 98	54

TABLE 8.—Invasive cervical cancer. Age-adjusted prevalence ratios of unsuspected cases detected at first screening of women age 20 and older*

Source of referral	Prevalence ratios per 1,000 women screened					
	Residents			Nonresidents		
	White	Negro	Total	White	Negro	Total
Private physician	1. 1	2. 2	1. 2	1. 0	*3. 0*	1. 1
Gailor-Gaston (City of Memphis hospitals)	3. 4	2. 0	2. 3	*1. 5*	*0*	0. 8
Other medical agencies	0. 8	*1. 5*	1. 0	*0*	*1. 5*	0. 4
Industrial clinics	1. 1	*0*	1. 1	*0*	*0*	*0*
Other special clinics	1. 4	0. 8	1. 4	*0*	*0*	*0*
Total	1. 3	1. 9	1. 5	0. 9	1. 5	1. 0

*Ratios are adjusted to the observed ratios for all residents 20 years of age and older, by the indirect method and are based on a total of 202 cases. Ratios in italics are based on less than 1,000 screenees.

Table 8 shows that no cases were found in some referral classes; in others, ratios in italics indicate extreme instability. Table 3 shows the small number examined in some referral groups. Where numbers are large enough to permit some interpretation, marked variation between groups may be noted. White private patients, the only nonresident group large enough to warrant comment, showed a prevalence ratio similar to the white-resident, private-physician referrals. Among white-resident participants, levels of prevalence varied, with a distinctly higher ratio (3.4 per 1,000) in Gailor-Gaston referrals than in those from private physicians (1.1 per 1,000). The special-clinic groups may show a somewhat greater prevalence than private referrals. Within the Negro resident group differences are not as notable, though prevalence seems to be lower among the groups from the special clinics. In spite of the small num-

ber of examinees in some classes, the table shows that, from a fundamental research standpoint, little significance should be attached to prevalence ratios unless something is known of the general characteristics of the women being examined. The variations in ratios are clearly not due to variations in application of the cytologic technique—such as use of vaginal pool versus cervical-scraping smears—nor to variation in cytologic or histologic interpretation. The only difference related to survey method was delayed diagnosis of 19 residents who had suspicious cytologic findings at first screening but did not have an adequate complete diagnosis until more than a year later. The major reason for the variation is likely to be unequal weighting of the different classes with respect to selective factors which are related to the occurrence of the disease. Consideration of some of the factors such as marital and parity status will be discussed in a later paper.

Tables 9 and 10 show prevalence ratios of intra-epithelial carcinoma (carcinoma *in situ*) in the same groupings as in tables 7 and 8 for invasive

TABLE 9.—Prevalence of unsuspected intra-epithelial carcinoma of cervix at first screening among Shelby County residents of age 20 and older, by race and age

Age	White		Negro	
	Number of cases	Ratio per 1,000	Number of cases	Ratio per 1,000
20–29	55	2. 08	56	3. 81
30–39	105	4. 31	60	6. 46
40–49	67	4. 47	23	3. 61
50–59	15	1. 86	8	2. 34
60–69	12	3. 18	4	2. 73
70 and over	4	2. 82	1	1. 89
Total	258	3. 26	152	4. 25

TABLE 10.—Intra-epithelial cervical carcinoma (carcinoma *in situ*); age-adjusted prevalence ratios of unsuspected cases detected at first screening of women age 20 and older*

Source of referral	Prevalence ratios per 1,000 women screened					
	Residents			Nonresidents		
	White	Negro	Total	White	Negro	Total
Private physician	3. 0	3. 6	3. 0	*2. 9*	*1. 8*	2. 9
Gailor-Gaston (City of Memphis hospitals)	5. 1	4. 5	4. 6	*8. 9*	*3. 6*	*6. 2*
Other medical agencies	3. 6	*1. 8*	1. 8	*7. 1*	*3. 6*	5. 7
Industrial clinics	3. 9	*4. 5*	4. 0	*3. 6*	*0*	*3. 6*
Other special clinics	3. 1	2. 1	3. 0	*7. 1*	*0*	*7. 1*
Total	3. 3	4. 2	3. 6	3. 4	3. 6	3. 4

*Ratios are age-adjusted to the ratios for all residents by the indirect method and are based on a total of 484 cases. (Of 486 cases, 2 were Negro patients under 20 years of age.) Ratios in italics are based on less than 1,000 screenees.

disease. It is clear there is less difference between the races in the prevalence of this lesion than that indicated for invasive disease and less variation among the classes shown in table 10, excluding the high ratios recorded in groups with relatively few examinees. Interpretation of the differences in magnitude of unsuspected invasive and intra-epithelial disease must be deferred until analysis of the rescreening experience can be presented.

EFFECT OF MASS SCREENING ON CERVICAL CANCER IN ENTIRE COMMUNITY

Prior to the beginning of the screening program a morbidity survey was conducted to determine the incidence of newly diagnosed invasive cancer of the uterus in the entire Memphis-Shelby County population. The purpose of this was to establish a base line of disease—both frequency and clinical stage at diagnosis—as it came to the attention of the medical profession immediately preceding cytologic screening. The rates resulting from this survey have been published (*11*). Since their publication, this type of morbidity survey has been extended through 1957, and data now include clinical stage of disease at diagnosis.

The prescreening morbidity which will be used in this and subsequent papers deals with data published (*11*) for 1950 and 1951, plus the cases for the first 6 months of 1952. This does not materially alter the published rates but does add to their stability. Because the details of these rates are more appropriate to a later paper dealing with incidence and prevalence rates, they will not be given here. During the prescreening period from January, 1950, to July, 1952, an average of 35.4 cases per year of invasive cervical cancer was diagnosed per 100,000 white females and 64.6 cases per 100,000 Negro females in Shelby County. Average annual rates per 100,000 females 20 years of age and older were 49.1 for the white and 97.7 for the Negro. The rate in Negro females is in the range of the highest recorded in similar surveys or in whole-population registries except for the extremely high rate (121.1) observed (*12*) among Puerto Rican women in New York City. If the population base upon which the latter rate is computed underestimates the true number of Puerto Rican women in New York City, or if there is a tendency for women with the disease to migrate to New York for medical care, this rate may be artificially inflated.

The rate observed in Memphis among white women is generally greater than rates similarly derived for white women in 10 metropolitan areas of the United States (*13*), in Connecticut (*14*), up-State New York (*15*), and in Denmark (*16*). The different age selection for the disease in Memphis, as compared with cervical cancer observed in some other localities, has already been noted (*7*).

In Memphis, before the introduction of the screening program, relatively little use had been made of female-genital, cytologic examination. The prescreening morbidity rates thus represent clinical incidence of invasive

disease as diagnosed prior to the time disease-detection practice was altered by large-scale use of the test. One way of determining the effect of this alteration on cancer detected subsequently is to apply the age-specific prescreening incidence rates to the entire population in the ensuing years. The result gives the number and clinical stage of cases which would have been expected if diagnostic practice had remained as before. The difference between the number and stage of cases expected and those actually observed reflects the net effect of mass screening on number and stage of diagnosed cases.

Table 11 presents a comparison of the cases expected under conditions of prescreening practice with those actually observed in the entire Shelby County population during the 5-year survey. In the white population, 383 cases were diagnosed compared to an expected 301, an excess of 27 percent. Among the expected 301 cases, 102 were stage I and 199 in a higher stage; 220 stage I cases were observed and only 163 cases in higher stages. The total of 315 cases observed in Negro women was only 3 percent over the expected 307, but the same kind of increase in stage I cases was evident. The ratio of observed-to-expected cases is higher at younger ages and in stage I cases than for older women and for higher stages.

The number of unsuspected cases among resident women at first screening was shown in table 7, with the number having clinical stage I disease. The greater representation of younger women in the screening program is reflected in the total morbidity (table 11), in the ratio of observed-to-expected cases which is strikingly higher at younger ages. The contribution of the survey participants to the increase in stage I disease is also evident.

Another test of the contribution of the survey to early diagnosis might be made if one could determine the average duration of the invasive process in the cases detected in the survey and compare this with duration in those diagnosed in the presurvey period. Although the data under consideration here do not permit quite this comparison, they do provide a basis for estimating how much longer invasive disease had been present in the cases diagnosed in the presurvey period. Average duration of disease is numerically the ratio of prevalence to incidence. Since the prevalence ratios of table 7 represent the proportion with invasive disease in the cytologically examined population, and presurvey morbidity represents the yearly incidence of newly diagnosed disease as it ordinarily came to medical attention in the presurvey period, the ratio of these rates represents the average duration of the invasive process from the time it was cytologically detected to the time it was ordinarily diagnosed. Computation of an age-adjusted ratio of these rates yields a value of 3.4 years for the white and 3.0 years for the Negro cases.

While values computed in this manner are subject to certain limitations which are too complex to be considered in this paper, they are believed to be sufficiently valid to conclude that cytologically detected cases were diagnosed *on the average* approximately 3 years earlier in their *invasive* process than the cases diagnosed in the community in the presurvey

TABLE 11.—Invasive carcinoma of the cervix. All new cases, stage I and higher stages diagnosed in Shelby County residents July 1, 1952, through June 30, 1957, compared with cases expected on the basis of presurvey morbidity incidence rates January 1, 1950, to June 30, 1952

Age	All cases			Stage I			Higher stages		
	Expected	Observed	Ratio observed/ expected	Expected	Observed	Ratio observed/ expected	Expected	Observed	Ratio observed/ expected
				White					
20–29	3. 9	12	3. 08	1. 9	12	6. 32	2. 0	—	—
30–39	41. 3	71	1. 72	17. 4	59	3. 39	23. 9	12	0. 50
40–49	82. 0	97	1. 18	32. 4	67	2. 07	49. 6	30	0. 60
50–59	64. 8	87	1. 34	17. 9	41	2. 29	46. 9	46	0. 98
60–69	56. 5	66	1. 17	18. 1	29	1. 60	38. 4	37	0. 96
70 and over	52. 2	50	0. 96	14. 2	12	0. 84	38. 0	38	1. 00
Total: 20 and over	300. 7	383	1. 27	101. 9	220	2. 16	198. 8	163	0. 82
				Negro					
20–29	3. 9	19	4. 87	1. 9	14	7. 37	2. 0	5	2. 50
30–39	43. 5	50	1. 15	15. 9	27	1. 70	27. 6	23	0. 83
40–49	89. 0	75	0. 84	16. 3	37	2. 27	72. 7	38	0. 52
50–59	77. 1	88	1. 14	8. 9	26	2. 92	68. 2	62	0. 91
60–69	53. 6	48	0. 90	9. 0	11	1. 22	44. 6	37	0. 83
70 and over	39. 7	35	0. 89	2. 3	4	1. 74	37. 4	31	0. 83
Total: 20 and over	306. 8	315	1. 03	54. 3	119	2. 19	252. 5	196	0. 78

period. Supporting this conclusion is the fact that at least half the survey-detected, stage I cases (table 11) showed truly preclinical lesions with microscopic invasive disease only.

In a different way, table 12 presents improvement in stage of disease at diagnosis. In the entire white population of the city and county 135 cases were diagnosed in the 2½-year presurvey period, of which 46 (34%) were diagnosed in clinical stage I. During the ensuing 5 years of the study, 383 cases were diagnosed in the entire white population, of which 220 (57%) were stage I cases. Of the 383 cases, 123 were unsuspected; 106 (86%) were in stage I. The remaining 260 of the 383 cases were either nonparticipants in the program or women with clinically suspected disease who had a cytologic examination in the course of their diagnosis. Of these, 114 (44%) were in stage I. This proportion of early cases represents about 30 percent improvement (from 34% in stage I to 44%) over that observed in the prescreening period and probably reflects a trend toward earlier diagnosis attributable at least in part to the screening program.

TABLE 12.—All new cases of invasive cervical carcinoma, diagnosed in Shelby County population between January 1, 1950, and June 30, 1957; number of cases and clinical stage of disease at diagnosis

	Presurvey period*		Survey period July 1952–June 1957					
	January 1950–June 1952		Total*		Unsuspected†		Other‡	
	White	Negro	White	Negro	White	Negro	White	Negro
			Number of cases					
All cases	*135*	*144*	*383*	*315*	*123*	*79*	*260*	*236*
Stage I	46	26	220	119	106	61	114	58
Higher stages	89	118	163	196	17	18	146	178
			Percent					
All cases	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*
Stage I	34	18	57	38	86	77	44	25
Higher stages	66	82	43	62	14	23	56	75

*All cases in entire population of county.

†All cases at and after first screening which were unsuspected at initial cytologic examination, among 122,009 residents with 1 or more satisfactory screenings.

‡All cases clinically suspected at diagnosis with or without cytologic examination.

A similar type of improvement in earlier detection of disease is evident (table 12) for the Negro patients. In percentages, the improvement in this respect was greater for the Negro than for the white, but since the proportion of Negro patients diagnosed in stage I during the presurvey period was so low, there is still room for improvement in this group.

Since the detection and treatment of most of these cases is recent, little importance may be attached to any statements regarding their survival.

Such analyses as are now possible, however, are fully consistent with the view that improvement in survival will be in proportion to the increase in the number of cases detected in an earlier stage of the disease. This phase of the study will be reported after adequate time has elapsed for follow-up analysis.

With regard to cancer of the uterine corpus, about the same number of cases was detected in the entire population during the study period as would have been expected had prescreening incidence rates prevailed. A distinct shift in white cases toward the younger ages, as well as a substantial increase in the proportion of females with localized disease at diagnosis, is another indication of the trend toward earlier diagnosis of uterine cancer which was accelerated by the publicity accompanying the survey. No shift in age selection was evident in the Negro population, but here also there was improvement in the proportion with localized disease.

In addition to the cases of invasive uterine cancer, a substantial number of women with intra-epithelial cervical carcinoma (carcinoma *in situ*) were identified. Although the records of treatment of these cases are not yet completely assembled, it is now known that at least 75 percent of the white and 50 percent of the Negro patients with intra-epithelial carcinoma have undergone radical treatment for their disease. If all cases of invasive cervical cancer originate in an *in-situ* lesion of long duration, then early detection and treatment should be followed by reduction in invasive disease in the population, after first screening. While no definitive statement is now possible as to whether or not this has happened, preliminary analysis of data bearing on it justifies raising the question as to whether this is really a significant sequence of events or, if it is, whether it is of a degree to be detected by the methods applied and the time presently elapsed in this study. The detailed epidemiological evidence bearing on this will be considered in a subsequent publication.

COMMENT

It should be emphasized that this program had two general objectives: (1) testing the cytologic technique as a mass screening device, and (2) assembling data bearing on the epidemiology and pathogenesis of cervical cancer. This report has dealt with some of the findings bearing on the first objective.

The function of a mass screening program is to detect suspects. The procedure is designed to select from a large population, by means of a relatively simple and inexpensive testing device, those individuals whose findings indicate a need for the more detailed and expensive study required for definitive diagnosis of disease. The vaginal aspiration technique was generally used in this study because it fulfilled a major requirement of a screening device, *i.e.*, large numbers of satisfactory specimens could be taken by trained nonprofessional persons in clinics established for this purpose. This is not intended to imply that smears made from the vaginal

pool are more likely to yield abnormal cells than smears obtained from cervical scrapings or aspiration. This is not the issue. It is merely that a procedure requiring cervical examination cannot be used on a mass scale because the physicians, whose time would be required, are not available.

In a mass screening program provision is made for reporting abnormal findings to physicians, with recommendations from the pathologist for further specific diagnostic investigation. It is inevitable that because of procrastination of the patient, or for other reasons, some cases detected by cytology were not completely diagnosed and treated as promptly as could be desired. Some had no tissue study; a few had biopsies not interpreted as positive and did not accept the further study recommended despite highly suspect cytologic findings. Counted in the unsuspected prevalence ratios (tables 7 and 8) are those meeting the arbitrary definition of a screening, *i.e.*, those cases with diagnosis completed within 1 year. Included are 10 cases of "false negative" cytology with positive tissue diagnosis, not related to cytology, within a year of the initial smear. These cases were not found by cytology but are included because they definitely were unsuspected at the time of the initial smear and met the screening definition. Not included are cases with completed diagnosis after more than a year and a few which were actually cancer, at the time of first screening, but were not diagnosed until events occurring after the arbitrary definition of a "screen" led to examination of additional sections of the original tissue. These will be taken into account in prevalence ratios used for epidemiological purposes but are not included here because the diagnosis at first screening was of no potential benefit to the patient.

The data presented here are a clear-cut tribute to the cytologic technique applied to vaginal aspiration smears and used as a mass screening device. Insofar as invasive cancer is concerned, the program—both directly and indirectly—benefited those individuals of the entire community who had cervical cancer, by earlier detection of the disease. Data bearing on the relationship of intra-epithelial carcinoma to invasive cervical cancer, involving study of the prevalence and incidence of the two lesions, will be presented in later communications.

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Specific Homograft Tolerance Induced by Successive Matings and Implications Concerning Choriocarcinoma [1,2]

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SUMMARY

Immunologic tolerance to the male antigen(s) in C57BL/An mice was induced in adult females of this strain by repeated matings with C57BL/An males. The tolerance was demonstrated by successful skin grafts from male donors to the females. The degree of tolerance was roughly proportional to the number of prior pregnancies. A partial tolerance may also have been induced by matings with sterile males. The observation that specific immunologic tolerance can be produced by successive matings of appropriate specificity offers an experimental model for explaining the apparent association of parity with the incidence of human choriocarcinoma and the apparent rarity of human erythroblastosis fetalis.—J. Nat. Cancer Inst. 25: 883–886, 1960.

RECENTLY Breyere and Barrett (*1*) presented data to show that allelograft [3] immunity could be mitigated if the recipient female had been repeatedly mated to a male possessing the specific histocompatibility antigens of the graft. Their data concerned the transplantation of a strain DBA/2 mouse sarcoma to multiparous females of the BALB/cAn strain which had borne litters by strain DBA/2 males. This result was somewhat surprising in view of the negative findings in experiments of similar type that were reported by Woodruff (*3*) and Medawar (*4*).

The contradictory reports suggested that an investigation be attempted along similar lines but with different antigens in a different genetic system. For this purpose we used the male antigen(s) originally discovered by Eichwald and Silmser (*5*) in C57BL mice. Tolerance in this system had been achieved previously by inoculation of antigen into newborn mice and by parabiosis (*6*). This antigen was known to be in our strain of C57BL/An mice, since the females regularly rejected male skin grafts, despite the fact that within-strain grafts were successful in all other circumstances (*7*).

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[2] This work was supported in part by grant C-4158 from the National Cancer Institute, National Institutes of Health, Public Health Service, and by grant 60-0353 from the State of Washington Initiative 171 Special Research Fund.

[3] The term *allelograft* has been proposed by Gorer for cases in which the graft differs from the recipient in *genetic* constitution. This usage avoids the confusion arising from the term *homograft* (*2*).

MATERIALS AND METHODS

Female mice from our C57BL/An colony were selected to form groups of comparable ages in the following categories: 4 to 6 previous litters, 1 to 3 previous litters, nulliparous mice that had been mated to sterile males [4], and virgins. The animals ranged in age from 4 to 16 months. The male which had been used for mating was in each case a littermate of the female concerned.

All mice were skin-grafted, with 3-month-old (approximately) C57BL/An males as skin-graft donors. The full thickness skin-grafting technique previously described was used except that surgical clips were substituted for the cotton suture material (*8*). This technique is used routinely in this laboratory and yields 90 to 100 percent successful isogenetic grafts. None of the mice were pregnant at the time of skin grafting and none had delivered a litter more recently than 2 weeks before. After grafting, the animals were kept in 3.5 × 5.0 cm., individual, hanging metal cages and were given water and Purina laboratory chow *ad libitum*.

The condition of the skin grafts was evaluated by gross observation twice weekly, and the grafts were rated on separate four-point scales as to size and hair growth. All ratings were made by the same individual who was unaware of the group to which any particular animal might belong.

As an additional control, 4 of the animals of the 4- to 6-litter group were challenged with an additional skin graft from a strain C3H/He male donor 27 days after the first skin graft.

RESULTS

The results with the C57BL/An male skin grafts are presented in table 1. It can be seen that there was increased tolerance to the male grafts associated with increased parity. Matings with sterile males produced a partial tolerance. The tolerance did not extend to the foreign-strain grafts (C3H/He) which were all sloughed rapidly in accordance with previous experience in this genetic system.

Those animals recorded in the table as having "good" grafts continued to have good grafts 3 months after grafting.

TABLE 1.—Results of skin grafts as recorded approximately 5 weeks after grafting

Number of litters	Number of mice	Number of "good" grafts*	Percent of "good" grafts
4–6	10	9	90
1–3	15	9	60
0 (mated)	15	7	47
0 (virgin)	15	0	0

*A "good" graft was rated 3+ or 4+ in regard to both size and hair growth.

[4] Sterile males are frequent in our colony. The basis of this sterility has not been investigated.

DISCUSSION

The results obtained extend the important observations of Breyere and Barrett (*1*) to another genetic system. That the tolerance was specific was suggested by the subsequent rejection of out-of-strain grafts by the tolerant females. A more critical control for evaluation of a possible nonspecific effect of previous pregnancies was not possible in this system; a control group of female mice, mated to females rather than to males, could not be used for obvious reasons. However, in view of the well-controlled studies of Breyere and Barrett, it is reasonable to believe by analogy that the results obtained were due to induction of a specific tolerant state.

The apparent difference in results between virgin and nonvirgin nulliparous mice raises interesting questions and requires further investigation. A possible explanation may be that the nulliparous nonvirgins might have actually experienced abortive pregnancies, but no definite information on this point is available.

That the results reported here and those of Breyere and Barrett (*1*) differ from those reported by Woodruff (*3*) and by Medwar (*4*) is best ascribed to the genetic differences in the systems used.

It is possible that a tolerance phenomenon in the human similar to that in the mouse may be a factor in the prevention of erythroblastosis fetalis, the incidence of which is very low even when antigenic differences between mother and child are marked.

That tolerance to certain histocompatibility antigens can be induced in the manner described may also have important implications with regard to human choriocarcinoma. In the female, choriocarcinoma is presumably derived from fetal trophoblast; therefore, the allelograft reaction should play a unique role in its biology (*9*). One would anticipate that this tumor, being in the nature of a genetically foreign tumor graft, would sometimes show spontaneous regression (which it does) and that it would be a tumor lending itself peculiarly well to chemotherapy. Hertz (*10*) has discussed the favorable results obtained with methotrexate in female choriocarcinoma as compared with results for the same lesion in the male whose tumor is not of foreign origin. His observations support the concept of an allelograft reaction in the females. Despite experimental evidence of others that there may be a partial nonspecific suppression of allelograft immunity *during* pregnancy (*11*), it can be postulated that most choriocarcinomas in women, which reach an extent allowing clinical recognition, are those in which the tumor has achieved some loss of "strain" specificity and/or those in which the patient has become partially or completely tolerant to the antigens in the trophoblast. That the latter might happen as the result of pregnancy is illustrated by the results of the animal experiments reported in this paper.

If this analysis of the situation in regard to choriocarcinoma is correct, it follows that female choriocarcinoma should be somewhat more deadly in multiparous women than in women after a first pregnancy. However,

since many antigens may be involved, not all of which might be present in each pregnancy, the increased tolerance would be detectable only by the statistical analysis of many hundreds of cases.

The epidemiologic analysis is complicated by the association of choriocarcinoma incidence with age, regardless of the possible role of parity. Park and Lees (*12*) found, in an analysis of 516 cases, a marked association between tumor incidence and patient age—a small part of which might have been accounted for by a direct effect of parity. A more recent study of choriocarcinoma in Asian countries also suggested the possibility of an etiologic association between parity and tumor incidence (*13*). These epidemiologic studies, although inconclusive, may have demonstrated the slight association predicted by a consideration of the experimental data. Thus, the epidemiology appears to be consistent with the concept that pregnancy-induced allelograft tolerance may be a factor in the biology of choriocarcinoma in the human female.

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Exchange of Radioactive Magnesium in Walker Carcinosarcoma 256: A Note [1,2]

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SUMMARY

Rats with Walker carcinosarcoma 256 were injected with radioactive magnesium, and then killed at intervals up to 7 hours. The exchange of tumor intracellular magnesium with plasma magnesium was complete in 4 hours. This rapid exchange is comparable with that in the liver, myocardium, and kidney in normal rats, but unlike the slower exchange in the skeletal muscle, brain, erythrocytes, bone, and testes.—J. Nat Cancer Inst. 25: 887–888, 1960.

A PREVIOUS publication from our laboratory has described the exchange of radioactive magnesium between the plasma and various tissues of the rat (*1*). It was found that tissues fall into two classes in this respect. In the liver, kidney, and myocardium, the specific activity of intracellular magnesium reaches that of the plasma magnesium within 3 hours, with a calculated turnover in 1.2 hours. In the skeletal muscle, brain, testes, and red cells, less than half the magnesium exchanges rapidly and the remainder equilibrates very slowly, with a turnover in 25 hours. The slow exchange in red cells precludes the otherwise reasonable possiblity that the blood flow through an organ determines the exchange rate. There is, however, some correlation with the level of metabolic activity in the tissues. It was of interest, therefore, to determine the exchange rate of magnesium in tumor tissue.

Walker carcinosarcoma 256 was implanted, by trocar, into the backs of male Wistar rats weighing 150 gm. After 3 weeks, 15 rats with non-necrotic tumors were selected and each was injected intraperitoneally with about 1 ml. of isotonic $Mg^{28}Cl_2$. The amount of isotope solution in each injection was measured exactly by weighing the syringe, before and after, on an analytical balance. The rats were killed at intervals up to 7 hours, and samples of the plasma, tumor tissue, and liver were taken for

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chemical and radioactive assay. At the time the animals were killed, the tumor weights were 2 to 5 percent of total body weights.

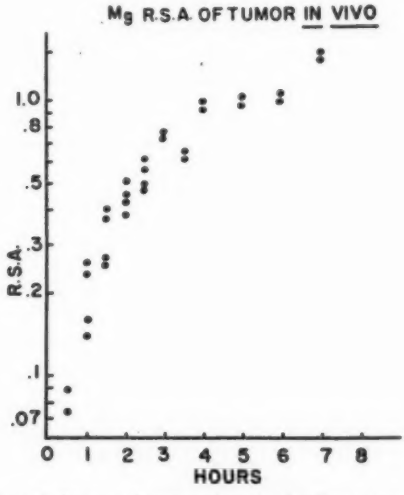
Tissue samples were first counted in a well-type scintillation counter, and the raw data corrected for isotopic decay and the amount of isotope injected per kg. body weight. After counting, the samples were ashed at 600° C. for 24 hours and the ash dissolved in 0.6 N HCl. These solutions were analyzed for magnesium by the method of Orange and Rhein (*2*). From these data the specific activity of the magnesium in each sample was calculated.

The degree of equilibration of the tissue magnesium with that of the plasma is given by the relative specific activity (rsa) which is the ratio:

$$\frac{\text{Tissue Mg specific activity}}{\text{Plasma Mg specific activity}}$$

When this value is equal to 1.0, equilibration is complete.

In text-figure 1, the plot of the rsa of magnesium in duplicate tumor-tissue samples shows that the exchange is rapid and complete within 4 hours. The equilibration closely resembles that of the "fast" organs in normal rats and is again consistent with the possibility that the exchange rate is faster in the metabolically more active tissues. The absolute magnesium content of the tumor tissues was 14.6 + 1.9 meq per kg. The exchange of magnesium in the livers of the tumor-bearing rats was not measurably different form that in normal rats.



TEXT-FIGURE 1.—Plot of the rsa of magnesium in duplicate tumor-tissue samples.

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Announcements

Establishment of a Coordinating Committee for Cell-Culture Collection

In response to the needs of virologists, cell biologists, and pathologists, the Advisory Panel on Viruses and Cancer of the National Advisory Cancer Council, on June 3, 1959, recommended the appointment of a committee to provide standards for mammalian cell lines. A national committee has recently been established under the chairmanship of Dr. Jerome T. Syverton. Members of the Committee are: Dr. John F. Enders, Children's Medical Center, Boston, Mass.; Dr. William A. Clark, American Type Culture Collection, Washington, D.C.; Dr. Lewis L. Coriell, South Jersey Medical Research Foundation, Camden, New Jersey; Dr. Cyril S. Stulberg, Child Research Center of Michigan, Detroit; Dr. Joseph W. Beard, Duke University School of Medicine, Durham, North Carolina; Dr. John G. Kidd, Cornell University Medical School, New York City; and Dr. Morgan Harris, University of California at Berkeley.

The Committee desires the support and active participation of cell culturists, cytologists, microbiologists, and scientists in all related disciplines so that an appropriate program may be set up. Dr. Coriell and Dr. Stulberg have agreed to establish the first two major centers for studying and banking cell strains. Dr. Clark will serve as director of a central repository and will accept any prototypes for frozen storage, which can be certified by the Committee, together with matching antiserums and seed stock for long-term preservation. Research will be done on preservation, storage procedures, and revival techniques.

Qualified investigators who wish to participate in this program should write Dr. Jerome T. Syverton, Chairman, Cell Culture Collection Coordinating Committee, Department of Bacteriology, University of Minnesota Medical School, Minneapolis 14, Minnesota.

Staff Appointments and Reorganization of Program Operations of the National Cancer Institute

Dr. Kenneth M. Endicott, who became Director of the National Cancer Institute July 1, 1960, has announced the appointment of three Associate Directors under whom program operations will be realigned in the areas of extramural research and training, field studies, and chemotherapy.

Dr. Ralph G. Meader, formerly Chief of the Research Grants Branch, has been designated Associate Director for Grants and Training. All of the Institute's grant

and training activities will be administered in different branches under his direction. He will have responsibility in particular for the expanding program of research on the viral etiology of cancer, and for comprehensive programs that may be authorized at designated research centers.

Dr. Michael B. Shimkin, formerly Chief of the Biometry Branch, has been designated Associate Director for Field Studies. Studies in biometry, epidemiology, environmental cancer, and diagnosis, including field investigations, will be administered in several branches under his direction.

Dr. Stuart M. Sessoms, Chief of the Cancer Chemotherapy National Service Center, has been designated Associate Director and will continue to head the Center.

The reorganization of activities may include the establishment of several new branches as well as the grouping of existing branches.

Dr. Endicott has also announced the following additional staff changes and reorganization:

Dr. Charles G. Zubrod, Clinical Director, has been assigned full responsibility for administration of intramural clinical research.

Dr. Carl G. Baker, Assistant Director, has been given responsibility for intramural nonclinical research.

Dr. Eli Nadel has been appointed an Assistant Director. Dr. Nadel was formerly Assistant to the Associate Director for Intramural Research, National Institutes of Health.

Manuscripts Approved for JNCI, August 15 to September 15, 1960

"DNA values (Feulgen microspectrophotometry) in the epithelium of the human ectocervix, normal and cancerous," by Bevan L. Reid and Sardool Singh.

"Long-term *in vitro* cultivation of some mouse ascites tumors: Ehrlich's ascites carcinoma," by Relda Cailleau and Françoise Costa.

"The *in vitro* behavior of a mouse lymphoid leukemia virus," by R. A. Manaker, P. C. Strother, A. A. Miller, and C. V. Piczak.

"Reducing bodies in the adrenal medulla fixed with Zenker-formol," by S. S. Spicer and Thelma B. Dunn.

"An intermittent perfusion and gas-exchange method for continuous cultivation of cells in the Rose chamber," by Willie D. Morgan and Clyde J. Dawe.

"Tumor formation with transplants of spleen or liver from mice with virus-induced leukemia," by Charlotte Friend and Jamil R. Haddad.

"Structural differences produced in mammalian cells by changes in their environment," by Max G. Menefee and Virginia J. Evans.

"The metabolism of mammary-gland slices from rats receiving progesterone and 3-methylcholanthrene," by E. Douglas Rees.

"The control of some cell contact reactions in tissue culture," by A. S. G. Curtis.

"The use of a particular derivative of hematoporphyrin in tumor detection," by Richard L. Lipson, Edward J. Baldes, and Arthur M. Olsen.

"Sex difference in liver tumorigenesis in rats ingesting *N*-2-fluorenylacetamide," by Herschel Sidransky, Billie P. Wagner, and Harold P. Morris.

"The male histocompatibility antigens in mouse endocrine tissues. Functional and histological evidence," by R. F. Gittes and P. S. Russell.

"Effect of heterologous, homologous, and autologous serums on human normal and malignant cells *in vitro*," by Jewel Plummer Cobb and Dorothy G. Walker.

"Lung cancer in World War I veterans: possible relation to mustard-gas injury and 1918 influenza epidemic," by Gilbert W. Beebe.

"Pathogenesis of a virus-induced leukemia in mice," by Thelma B. Dunn, John B. Moloney, Arleigh W. Green, and Betty Arnold.

"Attempted immunization against tumorigenesis," by Richmond T. Prehn.

"Virus of avian myeloblastosis. XVIII. Fixation of myeloblasts and phosphatase activity of loci of virus synthesis (viroplasts)," by D. Weinstein, J. R. Sommer, G. S. Beaudreau, C. Becker, R. A. Bonar, and J. W. Beard.

"Effect of Ehrlich ascites tumor on the mammary glands of mice of low and high mammary-cancer strains," by Bertie F. Argyris and Thomas S. Argyris.

"Bioassay on polyvinylpyrrolidones with limited molecular weight range," by Wilhelm C. Hueper.

"Method for the cytological detection of tumor cells in mouse blood *in vitro*," by Marvin M. Romsdahl, Elizabeth W. Chu, Roxane Hume, and Robert R. Smith.

"Bovine ocular squamous-cell carcinoma. IV. Tissue-culture studies of bovine ocular squamous-cell carcinoma and its benign precursor lesions," by John A. Sykes, Leon Dmochowski, E. Staten Wynne, and W. O. Russell.

ERRATA

Volume 25, Number 2, August 1960

In the paper entitled "Studies on Isoantigenic Variation in Mouse Lymphomas," by K. E. Hellström.

Page 254, table 11: *In* column "Name," 23d line, *for* LNSBS *read* LNSB.

Page 260, table 14: The last 2 lines of the last 3 columns are reversed and should *read:*

Antiserum		Percent of unstained cells			
Prepared in	Against	Control	Treated with antiserum 1:5	Cytotoxic index 1:5	Final titer
A.SW	A	48	52	0	Negative
A	A.SW	48	2	0.96	1.80

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